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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 9/00, 15/82, A01H 5/00, 5/10

A1

(11) International Publication Number: **WO 99/67367**

(43) International Publication Date: 29 December 1999 (29.12.99)

(21) International Application Number: PCT/US99/14022

(22) International Filing Date: 22 June 1999 (22.06.99)

(30) Priority Data:

60/090,240	22 June 1998 (22.06.98)	US
60/091,640	2 July 1998 (02.07.98)	US

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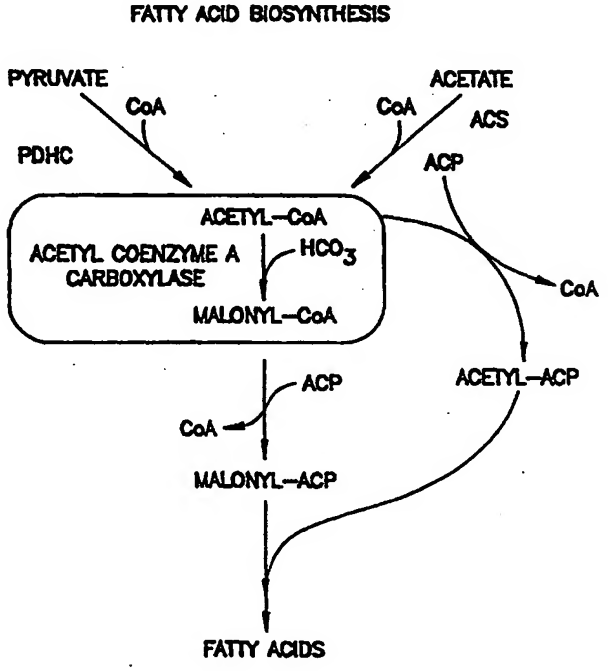
(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DNA ENCODING OAT ACETYL CoA CARBOXYLASE

(57) Abstract

The present invention provides the complete cDNA sequence of maize acetyl CoA carboxylase and methods for conferring herbicide tolerance and/or altering the oil content of plants by introducing and expressing a plant acetyl CoA carboxylase gene in plant cells. The method of imparting herbicide tolerance to a plant includes the steps of introducing an expression cassette encoding a plant acetyl CoA carboxylase or an antisense DNA sequence complementary to the sequence for a plant acetyl CoA carboxylase gene operably linked to a promoter functional in plant cells, into the cells of a plant tissue and expressing the plant acetyl CoA carboxylase gene in an amount effective to render the acetyl CoA carboxylase and/or plant cell tolerant to the herbicides. The method of altering the oil content in a plant includes the steps of introducing an expression cassette into plant cells and expressing the acetyl CoA carboxylase gene in an amount effective to alter the oil content of the cells. The expression cassette can also be introduced into other host cells to increase yield of a plant acetyl CoA carboxylase so that crystallized enzyme can be used to screen and identify other herbicides that bind to and inhibit the enzyme. Also provided is a partial DNA sequence encoding oat acetyl CoA carboxylase.



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DNA ENCODING OAT ACETYL CoA CARBOXYLASE

Background of the Invention

Acetyl CoA carboxylase (ACCase) is an enzyme involved in many important metabolic pathways in plant, animal and bacterial cells. The enzyme is especially important in fatty acid synthesis in plants and is sensitive to inhibition by some types of herbicides. Structurally, ACCases are biotinylated and are quite large enzymes consisting of one or more subunits. For example, most ACCases of animals, higher plants, and yeast are dimers of 420 to 700 kD native MW and contain subunits of 200 to 280 kD. Diatom and algal ACCases are 700 to 740 kD tetramers of 160 to 180 kD subunits. Bacterial ACCase consists of three dissociable proteins; biotin carboxylase (51 kD), biotin carboxyl carrier protein (22.5 kD), and biotin transcarboxylase (130 kD).

Acetyl CoA Carboxylase (ACCase) catalyzes the ATP-dependent formation of malonyl-CoA from acetyl-CoA and bicarbonate in animal, plant, and bacterial cells. Malonyl-CoA is an essential substrate for (i) de novo fatty acid (FA) synthesis, (ii) fatty acid elongation, (iii) synthesis of secondary metabolites such as flavonoids and anthocyanins, and (iv) malonylation of some amino acids and secondary metabolites. Synthesis of malonyl-CoA is the first committed step of flavonoid and fatty acid synthesis and current evidence suggests that ACCase catalyzes the rate-limiting step of fatty acid synthesis. Formation of malonyl-CoA by ACCase occurs via two partial reactions and requires a biotin prosthetic group:

(i)E-biotin + ATP + HCO_3 --> E-biotin-CO_2 + ADP + Pi

(ii)E-biotin-CO_2 + Acetyl-CoA --> E-biotin + malonyl-CoA

(NET)Acetyl-CoA + ATP + HCO_3 --> malonyl-CoA + ADP + Pi

In E. coli, these reactions are catalyzed by three distinct components; biotin carboxylase, biotin transcarboxylase, and biotin carboxyl carrier protein, which can be separated and yet retain partial activity. Plant and animal ACCases contain all three activities on a single polypeptide.

In plants, most ACCase activity is located in plastids of green and non-green plant tissues including leaves and oil seeds. Leaf ACCase activity is primarily located in mesophyll cells, but lesser amounts have been found in C-4

bundle sheath cells and in epidermal cells. The subcellular location of ACCase activity in epidermal cells is unknown, but since synthesis of very long-chain fatty acids (VLCFA) for formation of waxes, cutin, and suberin occurs on the endoplasmic reticulum (ER), malonyl-CoA might also be derived from a cytosolic ACCase. In contrast, rat ACCase is primarily cytosolic or associated with the outer mitochondrial membrane.

De novo fatty acid synthesis in chloroplasts involves successive 2-carbon additions to acetate, using malonate as the 2-C donor. All intermediates are attached to acyl carrier protein (ACP). Synthesis in plastids resembles that in E. coli in that the fatty acid synthesis complex can be dissociated into separate enzymes: β-ketoacyl-ACP synthase (KAS), β-ketoacyl-ACP reductase, β-hydroxyl-ACP dehydratase, and enoyl-ACP reductase, acetyl-CoA:ACP transacylase, and malonyl-CoA:ACP transacylase. A highly active KASIII isozyme catalyzes the condensation of acetyl-CoA and malonyl-ACP. Successive additions of malonyl-CoA to acyl-ACPs catalyzed by KAS I form C16 acyl-ACP, some of which is converted to C18 acyl-ACP by KAS II and then to C18:1-ACP. Fatty acid metabolism then diverges; de-esterification allows movement to the cytoplasm (eukaryotic path) where fatty acids may be further unsaturated and/or elongated by additions of malonyl-CoA in the ER. Alternatively, fatty acids are linked to glycerol-3-phosphate (prokaryotic path), further unsaturated, and used for synthesis of chloroplast lipids. A portion of cytoplasmic lipids returns to the chloroplast. The relative contributions of these two paths are species-specific but appear to be relatively flexible in mutants blocked in either path. In oil-storing organs such as cotyledons and monocot embryos, the triacylglycerides are stored in cytoplasmic oil bodies surrounded by a single unit membrane.

Condensation of malonyl-CoA with phenylpropionyl-CoAs or acetyl-CoA leads to synthesis of flavonoids, anthocyanins, or to polyacetates. Condensation is increased by light, elicitors, or pathogens and may be the rate-limiting step in synthesis of some phytoalexins. In addition to the secondary metabolites derived by de novo synthesis, malonyl conjugates of flavonoid glycosides, formed by malonyl-CoA:flavonoid glycoside malonyltransferase, D-amino acids and 1-amino-carboxyl-cyclopropane (ethylene precursor) are

found in plants. Malonylated compounds accumulate in vacuoles, probably after synthesis in the cytoplasm.

An important property of ACCase is the central role it plays in fatty acid synthesis and accumulation in plants and seeds. Available evidence supports the idea that ACCase activity is the rate-limiting step for de novo fatty acid synthesis in plants. High rates of ACCase activity in vitro parallel or slightly precede high rates of lipid deposition or [^{14}C]acetate incorporation into lipids in developing leaves and oil seeds. Significant changes in plant ACCase activity occur during chloroplast development and increase in ACCase activity correlates with lipid deposition in developing oil seeds. Turnham et al., Biochem. J., 212:223 (1883); and Beittenmiller et al., Plant Physiol., 100:923 (1992).

Among other properties, ACCase in most monocots is also inhibited by several herbicides. [^{14}C]acetate incorporation into maize lipids is strongly inhibited by fluazifop and sethoxydim due to inhibition of plastid ACCase. In barley, however, fluazifop had little effect on [^{14}C]acetate incorporation into very long-chain fatty acids. Since synthesis of very long-chain fatty acids occurs in the cytosol on the ER, and de novo fatty acid synthesis occurs in the plastids, cytosolic malonyl-CoA might be supplied by a herbicide insensitive ACCase isozyme.

There are three general mechanisms by which plants may be resistant to, or tolerant of, herbicides. These mechanisms include insensitivity at the site of action of the herbicide (usually an enzyme), rapid metabolism (conjugation or degradation) of the herbicide, or poor uptake and translocation of the herbicide. Altering the herbicide site of action from a sensitive to an insensitive form is the preferred method of conferring tolerance on a sensitive plant species. This is because tolerance of this nature is likely to be a dominant trait encoded by a single gene, and is likely to encompass whole families of compounds that share a single site of action, not just individual chemicals. Therefore, detailed information concerning the biochemical site and mechanism of herbicide action is of great importance and can be applied in two ways. First, the information can be used to develop cell selection strategies for the efficient identification and isolation of appropriate herbicide-tolerant variants. Second, it

can be used to characterize the variant cell lines and regenerated plants that result from the selections.

Tissue culture methods have been used to select for resistance (or tolerance) using a variety of herbicides and plant species (see review by Meredith and Carlson, 1982, in Herbicide Resistance in Plants, eds. Lebaron and Gressel, pp. 275-291, John Wiley and Sons, NY). For example, P. C. Anderson et al., in U.S. Patent No. 4,761,373, disclose the use of tissue culture methods to produce maize plants resistant to herbicidal imidazolidones and sulfonamides. The resistance is due to the presence of altered acetohydroxy acid synthase which is resistant to deactivation by these herbicides.

Certain 1,3-cyclohexanediones exhibit general and selective herbicidal activity against plants. One such cyclohexanedione is sethoxydim {2-[1-(ethoxyimino)-butyl]-5-[2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one}. Sethoxydim is commercially available from BASF (Parsippany, New Jersey) under the designation POAST™.

Other herbicidal cyclohexanediones include clethodim, (E,E)-(±)-2-[1-[[(3-chloro-2-propenyl)oxy] imino]propyl]-5-[2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one; available as SELECT™ from Chevron Chemical (Valent) (Fresno, California); cloproxydim, (E,E)-2-[1-[[(3-chloro-2-propenyl)oxy]imino] butyl]-5-[2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one; available as SELECTONE™ from Chevron Chemical (Valent) (Fresno, California); and tralkoxydim, 2-[1-(ethoxyimino)propyl]-3-hydroxy-5-mesitylcyclohex-2-enone, available as GRASP™ from Dow Chemical USA (Midland, Michigan).

For purposes of reference in the present specification, the herbicides described in the two preceding paragraphs and other structurally related herbicidal compounds, are collectively referred to as the cyclohexanedione family of herbicides.

Certain aryloxyphenoxypropanoic acids exhibit general and selective herbicidal activity against plants. In these compounds, the aryloxy group may be phenoxy, pyridinyloxy or quinoxalinyl. One such herbicidal aryloxyphenoxypropanoic acid is haloxyfop, {2-[4-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]oxy]phenoxy]- propanoic acid}, which is available

as VERDICT™ from Dow Chemical USA (Midland, Michigan). Another is diclofop, {(±)-2-[4-(2,4-dichlorophenoxy)-phenoxy]propanoic acid}, available as HOELON™ from Hoechst-Roussel Agri-Vet Company (Somerville, New Jersey).

Other members of this family of herbicides include fenoxyaprop, (±)-2-[4-[(6-chloro-2-benzoxazolyl)oxy] phenoxy]propanoic acid; available as WHIP™ from Hoechst-Roussel Agri-Vet Company (Somerville, New Jersey); fluazifop, (±)-2-[4-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenoxy]propanoic acid; available as FUSILADE™ from ICI Americas (Wilmington, Delaware); fluazifop-P, (R)-2-[4-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenoxy]propanoic acid; available as FUSILADE 2000™ from ICI Americas (Wilmington, Delaware); and quizalofop, (±)-2-[4[(6-chloro-2-quinoxalinyl)-oxy]phenoxy]propanoic acid; available as ASSURE™ from E. I. DuPont de Nemours (Wilmington, Delaware).

For purposes of reference in the present specification, the herbicides referred to in the two preceding paragraphs and other structurally related herbicidal compounds, are collectively referred to as herbicidal aryloxyphenoxypropanoic acids.

Thus, there is a need for methods to develop plants that are resistant or tolerant to herbicides. There is also a need to increase the oil and/or fatty acid content of the plants and seeds, as well as for methods to increase their resistance to herbicides. Moreover, there is a need to identify and clone genes important in conferring herbicide tolerance and in increasing the oil content of plants.

Summary of the Invention

The present invention provides an isolated and purified DNA molecule comprising a DNA segment encoding a plant acetyl CoA carboxylase such as a monocot acetyl CoA carboxylase, e.g., a maize or oat acetyl CoA carboxylase, and methods for conferring herbicide tolerance and/or altering the oil content of plants by introducing and expressing a plant acetyl CoA carboxylase gene in the plant cells. The DNA molecule encoding a plant acetyl CoA carboxylase can encode an unaltered plant acetyl CoA carboxylase or an

altered plant acetyl CoA carboxylase substantially tolerant to inhibition by cyclohexanedione or aryloxyphenoxypropanoic acid herbicides as well as encoding an antisense DNA sequence that is substantially complementary to a plant acetyl CoA carboxylase gene or to a portion thereof. A DNA molecule of the invention can also further comprise an amino terminal plant chloroplast transit peptide sequence operably linked to the plant acetyl CoA carboxylase gene. The DNA molecule of the invention preferably encodes the plastidic form of plant acetyl CoA carboxylase. The invention also provides for probes, e.g., a probe which is detectably labeled or which binds to a detectable label, or primers, e.g., a single-strand DNA molecule having at least seven nucleotide bases, that hybridize to a DNA encoding a plant acetyl CoA carboxylase, e.g., a DNA molecule comprising SEQ ID NO:23 or its complement. For example, the present invention provides an isolated and purified DNA molecule of at least seven nucleotide bases which hybridizes under high stringency conditions to a DNA molecule comprising a DNA segment encoding a plant acetyl CoA carboxylase and provides a hybridization probe comprising an isolated and purified DNA molecule of at least seven nucleotide bases, which is detectably labeled or which binds to a detectable label, which DNA molecule hybridizes under high stringency conditions to the non-coding strand of a DNA molecule comprising a DNA segment encoding a plant acetyl CoA carboxylase. High stringency conditions are defined as: hybridization at 65°C for at least 16 hours in 5xSSC, 1x Denhardt's solution, 50 mM Tris-HCl, pH 8, 0.2% SDS, 10 mM EDTA, 0.1 mg/ml salmon sperm DNA, followed by washing twice for 5 minutes in 2xSSC, 0.5% SDS at 25°C, once for 10 minutes in 0.2xSSC, 0.1% SDS at 25°C and twice for 30 minutes in 0.2xSSC, 0.1% SDS at 65°C.

The method of imparting cyclohexanedione or aryloxyphenoxypropanoic acid herbicide tolerance to a plant includes the steps of introducing a chimeric or recombinant DNA molecule comprising a gene coding for a plant acetyl CoA carboxylase or an altered or a functional mutant thereof operably linked to a promoter functional in a plant cell into cells of a susceptible plant, and regenerating the transformed plant cells to provide a differentiated plant. The promoter can be an inducible or tissue specific promoter or provide for overexpression of at least about a 2-fold amount of a

native plant acetyl CoA carboxylase. The functional linkage of a promoter to the chimeric DNA molecule results in an expression cassette. Expression of the chimeric DNA molecule is in an amount effective to render the acetyl CoA carboxylase and/or the plant tissue substantially tolerant to the herbicides relative to the native acetyl CoA carboxylase present in said plant. Herbicide tolerance can be achieved in the plants by at least two methods, including increasing the level of gene expression of a native or unaltered acetyl CoA carboxylase, or by introducing an altered gene coding for an acetyl CoA carboxylase that is less sensitive to herbicide inhibition. The level of gene expression can be increased by either combining a plant acetyl CoA carboxylase gene with a promoter that provides for a high level of gene expression, such as a 35S cauliflower mosaic virus promoter (CaMV), or by introducing multiple copies of the gene into the cell so that the multiple copies of the gene are integrated into the genome of transformed plant cells. The preferred plant cells into which to introduce the expression cassette of the invention, to achieve herbicide tolerance, are monocot plant cells. Preferably, the cells are not oat cells. Once transformed cells exhibiting herbicide tolerance are obtained, transgenic plants and seeds can then be regenerated therefrom, and evaluated for stability of the inheritance of the herbicide tolerance trait.

The invention also provides a method for altering, e.g., lowering or raising, the oil content in a plant. As described hereinbelow, a locus having a major effect on oat (*Avena sativa* L.) groat oil content was located on linkage group 11 by quantitative trait locus (QTL) dissection methods. A partial oat acetyl-CoA carboxylase (ACCase) cDNA with >84% identity to plastidic ACCase sequences from wheat or maize was linked to this locus. Similar QTL and ACCase placements were obtained with two recombinant inbred populations ($n \geq 137$) with one common parent. The locus on linkage group 11 linked to the ACCase locus accounted for up to 48% of the phenotypic variance for groat oil content. Other loci were identified which accounted for an additional 14% of the phenotypic variance. The major locus plus additional QTLs represent three potentially homoeologous genomic segments in oat that can be followed by as few as three RFLP markers to produce a large shift in groat oil content. Oat acetyl CoA carboxylase DNA is also useful in molecular marker-assisted

selection to facilitate breeding high- or low-oil oat, genetic dissection of the groat oil trait, and to identify specific genes with large effects whose activity may be manipulated via genetic transformation.

Thus, the invention provides a method which includes the steps of introducing a chimeric or recombinant DNA molecule comprising a gene coding for a plant, e.g., oat, acetyl CoA carboxylase or an altered or a functional mutant thereof operably linked to a promoter functional in a plant cell into the cells of plant tissue and expressing the gene in an amount effective to alter the oil content of the plant cell. An alteration in oil content can include a change in total oil content over that normally present in that type of plant cell, a change in the location of the oil in the plant, plant tissue, seed or plant part, and/or a change in the type of oil present in the cell. For example, low-oil oat includes, but is not limited to, an oat plant, plant part, plant tissue or groat having about 8 to about 40, preferably about 8 to about 30, and more preferably about 20 to about 30, percent dry weight of oil relative to the corresponding parental, e.g., nontransformed or inbred, oat plant, plant part, plant tissue or groat. High-oil oat includes, but is not limited to, an oat plant, plant part, plant tissue or groat having about 2 to about 8, preferably 1 to about 4, and more preferably about 2 to about 4, percent dry weight of oil relative to the corresponding parental oat plant, plant part, plant tissue or groat.

An alteration in oil content in the plant cell can be achieved by at least two methods including:

(1) an increase or decrease in expression of an unaltered or altered plant acetyl CoA carboxylase gene; or

(2) by introducing an unaltered, altered or functional mutant plant acetyl CoA carboxylase gene.

The level of gene expression of an unaltered plant acetyl CoA carboxylase gene can be increased by either combining an unaltered plant acetyl CoA carboxylase with a promoter that provides for a high level of gene expression, or by introducing multiple copies of an expression cassette into cells so that multiple copies of the gene are integrated into the genome. When an altered or a functional mutant plant acetyl CoA carboxylase gene codes for an enzyme that exhibits an increase in specific activity, it can lead to an increase in total oil

content of the plant cell. When an altered or a functional mutant acetyl CoA carboxylase gene codes for an enzyme having a decrease in specific activity, it may lead to a decrease in the total oil content of the plant cell. Preferably, the expression cassette is introduced into dicot plants such as soybeans, canola, and sunflower. In an especially preferred version, transformed cells exhibiting about a 1.2- to 5-fold increase in total oil content and/or expression or specific activity of acetyl CoA carboxylase are selected for and used to generate transgenic plants and seeds exhibiting a substantial increase in oil content. A substantial increase in oil content depends on the oil content normally present in the plant or seed and can range from about a 1.2 to a 20-fold increase.

The invention also provides for a method of producing plant acetyl CoA carboxylase in a host cell. The method includes the steps of introducing an expression cassette comprising a chimeric gene encoding a plant acetyl CoA carboxylase or an altered or a functional plant acetyl CoA carboxylase operably linked to a promoter into a host cell and expressing the gene in an amount sufficient to permit crystallization of the plant acetyl CoA carboxylase. An expression cassette can include a promoter that is functional in either a eukaryotic or a prokaryotic cell. Preferably, the expression cassette is introduced into a prokaryotic cell, such as E. coli, that is routinely used for production of recombinantly produced proteins. Recombinantly produced and crystallized plant acetyl CoA carboxylase can then be used to identify other herbicides and that bind to and inhibit acetyl CoA carboxylase in plants. In addition, the availability of large amounts of purified enzyme can permit the screening of the efficacy of such herbicides in terms of their ability to bind to, or otherwise inhibit, the activity of the enzyme.

The present invention also provides a method of introducing an exogenous plant acetyl CoA carboxylase gene into a host cell comprising transforming host cells *in vitro* with an expression cassette comprising a chimeric or recombinant DNA molecule encoding a plant acetyl CoA carboxylase gene operably linked to a promoter functional in the host cell, expanding the transformed host cells *in vitro*, and identifying a transformed host cell which expresses the chimeric DNA molecule.

Further provided is a method to identify a plant, e.g., an oat plant, having altered characteristics, e.g., altered groat oil content. The method comprises contacting a probe comprising at least a portion of a nucleic acid sequence encoding oat acetyl CoA carboxylase with a sample from an oat plant in an amount sufficient to form complexes, wherein the sample comprises plant genomic nucleic acid. The amount of complex formation is then detected or determined. Preferably, the plant cells in the sample are monocot plant cells, e.g., wheat, rice, maize, barley, millet (e.g., foxtail or finger), sorghum, sugarcane, rye, oat or timothy cells. Also preferably, the DNA molecule encodes a polypeptide comprising SEQ ID NO:24, e.g., a DNA molecule comprising SEQ ID NO:23. Thus, the invention provides a nucleic acid fragment useful in marker-assisted breeding programs such as those which identify plants having altered groat oil content or composition.

Also provided is an expression cassette comprising an endosperm-specific transcriptional control region, e.g., the Dy10 or zein transcriptional control region, operably linked to a DNA encoding a plant acetyl CoA carboxylase, or subunit thereof. Other endosperm-specific promoters are also useful in the compositions and methods of the invention. These promoters include, but are not limited to, ltr1 promoter, a synthetic promoter with endosperm box of HOD (Vincentecarbajosa et al., Plant J., 13, 629 (1998)); rice glutelin gene promoter (Sindhu et al., Plant Sci., 130, 189 (1997); Yoshihara et al., Plant Sci., 121, 63 (1996)); barley alpha-amylase inhibitor subunit promoter (Gross et al., Plant Mol. Biol., 34, 331 (1997)); gamma zein promoter (Towent et al., Plant Mol. Biol., 34, 139 (1997)); maize opaque-2 locus promoter (Rossi et al., J. Biol. Chem., 272, 13758 (1997); Gallusci et al., Mol & Gen. Genet., 244, 391 (1994)); the zmGBS, zmZ27, osAGP or osGT1 promoter (Russell et al., Transgenic Res., 6, 157 (1997)); alpha globulin promoter (Nakase et al., Plant Mol. Biol., 33, 513 (1997)); hordein promoter (Sorenson et al., Mol. & Gen. Genet., 250, 750 (1996)); Viciafaba legumin and vicilin storage protein gene promoter (Wobus et al., J. Plant Physiol., 145, 592 (1995)); rice waxy gene promoter (Hirano et al., Plant & Cell Physiol., 36, 37 (1995)); oat globulin gene osglo5 promoter (Schubert et al., Plant Mol. Biol., 26, 203 (1994)); napA

promoter (Stalberg et al., Plant Mol. Biol., 23, 671 (1993)); and rice prolamin gene promtoer (Zhou et al., Science in China, 36, 1307 (1993)).

The term "consists essentially of" as used with respect to the present DNA molecules is defined to mean that a major portion of the nucleotide sequence encodes an ACCase, and that nucleotide sequences are not present which encode proteins other than ACCase or functional equivalents thereof.

Brief Description of the Figures

Figure 1: A schematic depiction of the fatty acid biosynthesis pathway in plants.

Figure 2: A graph depicting the effect of sethoxydim on the growth of mutant maize callus.

Figure 3: A graph depicting the shoot length growth of maize seedlings seven days after treatment with sethoxydim.

Figure 4: A graph depicting the shoot length growth of maize seedlings fourteen days after treatment with sethoxydim.

Figure 5: Total soluble and biotinylated polypeptides in ACCase purification fractions from seedling leaves of maize inbred A619. Proteins were separated by SDS-PAGE in 7.5% gels and then silver-stained (Panel A). An identical gel was Western-blotted and a longitudinal section of each lane was probed with avidin (Panel B). Lanes were 1: crude (10 μg); 2: $(NH_4)_2SO_4$ (10 μg); 3: S-300 (5 μg); 4: Blue Sepharose (2 μg); 5: Mono-Q ACCase II (5 μg); and 6: Mono-Q ACCase I (5 μg). Diagonal lines between lanes indicate position of molecular weight markers shown on the left.

Figure 6: Immunoprecipitation of ACCase activity from B73 leaf, embryo, endosperm, and BMS suspension cultured cells. Equal activities (0.58 nmol min^{-1}) were incubated with 16 μL serum (immune plus preimmune), immune complexes were precipitated with Protein A-agarose, and ACCase activity remaining in the resulting supernatant fraction was measured relative to the preimmune serum control.

Figure 7: Herbicide inhibition of acetyl-(AcCoA) or propionyl-CoA (Prop-CoA)-dependent $H^{14}CO_3$-incorporation into acid-stable

product by ACCase I and II Mono-Q fractions. Activities in the presence of haloxyfop (1 μM) are expressed relative to the minus herbicide control.

Figure 8: Comparison of the peptide sequence of maize cDNA clones #15-14 and #18-5 with chicken ACCase. The approximate locations of the biotin carboxylase, biotin binding site, and biotin transcarboxylase functional domains are indicated for the chicken sequence. The percentages of amino acid identity are indicated by cross-hatched boxes for the maize coding sequence. Regions of genomic DNA Type I and Type II clone sequences that align with cDNA #18-5 are indicated by solid heavy lines. The approximate locations of subclone #28 and #16 from genomic Type I and subclone #34 from genomic Type II clones are indicated.

Figure 9: Northern blot of total RNA from leaf, immature embryo and endosperm tissue (16 days after pollination), and Black Mexican Sweet corn (BMS) cells. Lanes contain 10 μg total RNA and were probed with the 2 kb EcoRI fragment of lambda clone #15-14.

Figure 10: DNA sequence (SEQ ID NO. 1) of a 2 kb EcoRI fragment of lambda clone #15-14 including a portion of a maize ACCase gene located at bases 2883 to 83 from the 3′ stop codon.

Figure 11: Graph of ACCase activity during seed development in two high oil soybean cell lines and one low oil soybean cell line.

Figure 12: Cloning strategy to obtain the complete coding sequence of the maize ACCase gene.

Figure 13: DNA sequence (SEQ ID NO:5) of a 7470 base pair cDNA of a maize ACCase gene. (Genbank Accession No. U19183).

Figure 14: Predicted amino acid sequence of the complete ACCase gene of maize (SEQ ID NO:6).

Figure 15: Restriction map of ACCase genomic clones.

Figure 16: Partial nucleotide sequence of a Type A_1 ACCase genomic clone (SEQ ID NO:12).

Figure 17: Partial nucleotide sequence of clone 5A, a Type A ACCase genomic clone (SEQ ID NO:13).

Figure 18: Partial nucleotide sequence of six Type A_2 ACCase genomic clones (A-F) (SEQ ID NOs 14, 15, 16, 17, 18, and 19).

Figure 19: Partial nucleotide sequence of three Type B ACCase clones (SEQ ID NOs 20, 21 and 22).

Figure 20: SDS/PAGE analysis of chloroplast importation of ^{35}S-labeled ACCase polypeptides. (A) Pea and maize chloroplasts incubated with ^{35}S-labeled ACCase polypeptides for 30 minutes. (B) A time course analysis of the importation of ^{35}S-labeled ACCase polypeptides into maize chloroplasts.

Figure 21: Partial DNA sequence encoding oat ACCase (SEQ ID NO:23, Genbank Accession No. AF072737).

Figure 22: Partial amino acid sequence of an oat ACCase (SEQ ID NO:24).

Figure 23: Scan of test statistics generated by MQTL for the two recombinant inbred populations. A and B are the main effect and QTL × E interaction graphs for the Kanota X Ogle (KO) population, respectively. C and D are the main effect and QTL × E interaction graphs for the Kanota X Marion (KM) population, respectively. Thresholds for type I error rate of 5% after 1000 replications are reported above the graphs.

Figure 24: Histogram of % groat oil concentration for the mean of all environments relative to the number of recombinant inbred lines (RILs) with each parental genotype for the XaccaseA locus. A) The distribution for the Kanota X Ogle population, and B) distribution for the Kanota X Marion population.

Figure 25: Phylogram of ACCases.

Detailed Description of the Invention

The present invention provides a DNA molecule encoding a plant acetyl CoA carboxylase gene and methods for conferring herbicide tolerance and/or altering the oil content of plants by introducing and expressing a plant acetyl CoA carboxylase gene in the plant cells. In plants, acetyl CoA carboxylase plays a central role in regulating fatty acid synthesis and in the sensitivity of monocots to cyclohexanedione or aryloxyphenoxypropanoic acid herbicides.

In accord with the present invention, a plant acetyl CoA carboxylase gene is identified, isolated and combined with a promoter functional

in a plant cell to provide a recombinant expression cassette. A plant acetyl CoA carboxylase gene can be introduced and expressed in a plant cell. Depending on the type of plant cell, the level of gene expression, and the activity of the enzyme encoded by the gene, introduction of a plant acetyl CoA carboxylase gene into the plant cell can confer herbicide tolerance and/or alteration of the oil of the plant cell.

In monocots, an exogenously introduced plant acetyl CoA carboxylase gene can be expressed at a level effective to render the cells of the plant tissue substantially tolerant to cyclohexanedione or aryloxyphenoxypropanoic acid herbicide levels which normally inhibit a native or endogenous acetyl CoA carboxylase. A native acetyl CoA carboxylase is an enzyme that is normally encoded and expressed in the plant cell prior to transformation. An exogenously introduced plant acetyl CoA carboxylase gene is a gene which has been isolated and amplified from either the same or different type of cell. Exogenous introduction and expression of a plant acetyl CoA carboxylase gene in both monocots and dicots can result in alteration of the oil content and quality of plant tissue and seeds. Exogenous introduction and expression in a host cell, such as a bacteria, can provide for sufficient amounts of plant acetyl CoA carboxylase to allow for crystallization and isolation of the enzyme. Crystallized plant acetyl CoA carboxylase is useful to identify other herbicides that bind to and can inhibit plant acetyl CoA carboxylases. The enzyme could also be used to screen potential herbicidal compounds for efficacy.

A. Formation of an Expression Cassette

An expression cassette of the invention can comprise a chimeric DNA molecule encoding a plant acetyl CoA carboxylase gene or an altered or functional mutant thereof operably linked to a promoter functional in a plant cell. The gene can code for a plant acetyl CoA carboxylase that is substantially tolerant to herbicides, preferably cyclohexanedione and/or aryloxyphenoxypropanoic acid herbicides. An expression cassette of the invention can also include an antisense DNA sequence that is substantially complementary to an acetyl CoA carboxylase gene or a portion thereof operably linked to a promoter functional in a plant cell.

1. **Isolation and Identification of a Gene Coding for a Plant Acetyl CoA Carboxylase**

A gene encoding a plant acetyl CoA carboxylase can be identified and isolated by standard methods, as described by Sambrook et al., Guide to Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY (1989). The gene can be obtained either from monocot or dicot plant cells. When the gene encoding a plant acetyl CoA carboxylase is obtained from a dicot plant, the enzyme encoded by the gene exhibits tolerance to cyclohexanedione or aryloxyphenoxypropanoic acid herbicides. The gene can also be obtained from herbicide-tolerant maize cell lines, prepared as described in U.S. Patent No. 5,162,602.

A gene encoding a plant acetyl CoA carboxylase can be identified by screening of a DNA or cDNA library generated from plant cells. Screening for DNA fragments that encode all or a portion of the gene encoding a plant acetyl CoA carboxylase can be accomplished by complementation of an auxotrophic mutant of acetyl CoA carboxylase in E. coli (fabE) (Bachman, Microbiological Reviews, 47:180 (1983)) or yeast (accl) (Michionada, Eur. J. Biochem., 111:79 (1980)) or by screening of plaques for binding to antibodies that specifically recognize a plant acetyl CoA carboxylase. DNA fragments that can restore ACCase activity in E. coli or yeast and/or plaques carrying DNA fragments that are immunoreactive with antibodies to a plant ACCase can be subcloned into a vector and sequenced and/or used as probes to identify other cDNA or genomic sequences encoding all or a portion of a plant acetyl CoA carboxylase gene.

Specific examples of cDNA sequences encoding a portion of a plant acetyl CoA carboxylase gene include DNA fragments that include a DNA sequence that substantially corresponds to the coding sequence for the transcarboxylase active site of a plant acetyl CoA carboxylase, DNA fragments that include a DNA sequence that substantially corresponds to a coding sequence for the biotin binding site of a plant acetyl CoA carboxylase, a DNA fragment encoding the 5′ transcriptional start sequence of a plant acetyl CoA carboxylase gene, and a DNA fragment encoding the 3′ transcriptional stop sequence for the acetyl CoA carboxylase gene. Substantially corresponding DNA sequences

share about 90% to about 100% DNA sequence homology. Especially preferred cDNA probes can be obtained from lambda clone #18-5 which include DNA sequences corresponding to the transcarboxylase active site domain and the biotin binding site domain. Lambda clone #18-5 includes EcoRI subclones of 3.9 kb, 1.2 kb, or 0.23 kb. Lambda subclone #18-5I is an 3.9 kb EcoRI subclone. The lambda subclone #18-5I has been deposited with the American Type Culture Collection, Rockville, MD, and given Accession No. 69236.

In a preferred version, a plant acetyl CoA carboxylase gene is identified and isolated from an herbicide tolerant maize cell line prepared as described in Example II. A cDNA library can be prepared by oligo dT priming. Plaques containing DNA fragments can be screened with antibodies specific for maize acetyl CoA carboxylase. DNA fragments encoding a portion of an acetyl CoA carboxylase gene can be subcloned and sequenced and used as probes to identify a genomic acetyl CoA carboxylase gene. DNA fragments encoding a portion of a maize acetyl CoA carboxylase can be verified by determining sequence homology with other known acetyl CoA carboxylases, such as chicken or yeast acetyl CoA carboxylase, or by hybridization to acetyl CoA carboxylase specific messenger RNA. Once DNA fragments encoding portions of the 5′, middle and 3′ ends as well as the transcarboxylase active site or biotin binding site of a plant acetyl CoA carboxylase are obtained, they can be used to identify and clone a complete genomic copy of a maize acetyl CoA carboxylase gene.

To isolate a complete copy of a maize acetyl CoA carboxylase gene, a maize genomic library can then be probed with cDNA probes prepared as described above. Portions of the genomic copy or copies of a plant acetyl CoA carboxylase gene can be sequenced and the 5′ end of the gene are identified by standard methods including either DNA sequence homology to other acetyl CoA carboxylase genes or by RNAase protection analysis, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, New York (1989). Once portions of the 5′ end of the gene are identified, complete copies of a plant acetyl CoA carboxylase gene can be obtained by standard methods, including by cloning or by polymerase chain reaction (PCR) synthesis using oligonucleotide primers complementary to the DNA sequence at the 5′ end of the gene. The presence of an isolated full-length

copy of a plant acetyl CoA carboxylase gene can be verified by hybridization, partial sequence analysis, or by expression of a plant acetyl CoA carboxylase enzyme. The maize acetyl CoA carboxylase gene cloned and expressed from a maize herbicide tolerant cell line can be assessed for tolerance to cyclohexanedione or aryloxyphenoxypropanoic acid herbicides by standard methods, as described in Example I.

An expression cassette of the invention can also contain an antisense DNA sequence. A antisense DNA sequence is a sequence that is substantially complementary to all or a portion of a coding sequence of a plant acetyl CoA carboxylase gene. A substantially complementary sequence has about 90% to about 100% DNA sequence homology with that of the coding sequence of all or a portion of a plant acetyl CoA carboxylase. The antisense DNA sequence when expressed can act to inhibit the synthesis and expression of a native plant acetyl CoA carboxylase. Antisense sequences are preferably about 200 to 1000 nucleotides long in order to provide sufficient inhibition of synthesis and/or expression of a native acetyl CoA carboxylase. The inhibition of acetyl CoA carboxylase synthesis and gene expression by antisense DNA sequences can be confirmed in a transformed plant cell by standard methods for measuring the presence and/or activity of the enzyme such as described in Examples I and V.

An expression cassette of the invention can also include a functional mutant of a plant acetyl CoA carboxylase gene. Mutants of a plant acetyl CoA carboxylase gene are substantially homologous to a plant acetyl CoA carboxylase gene and are functional if the acetyl CoA carboxylase expressed retains significant enzyme activity. A mutant substantially homologous to a plant acetyl CoA carboxylase can share about 90% to 99.99% DNA sequence with that gene. For example, a mutant acetyl CoA carboxylase gene can code for a herbicide tolerant acetyl CoA carboxylase, or for an acetyl CoA carboxylase with altered substrate specificity so that the total amount of oil content in the plants or seeds is increased, or for an enzyme with an altered substrate specificity so that synthesis of secondary metabolites such as flavonoids or anthocyanins is decreased. A preferred mutant is a gene coding for an acetyl CoA carboxylase

that is substantially tolerant to cyclohexanedione or aryloxyphenoxypropanoic acid herbicide

Altered or functional mutants of a gene coding for a plant acetyl CoA carboxylase can be obtained by several methods. The alteration or mutation of the ACCase gene can be accomplished by a variety of means including, but not limited to, the following methods.

1. spontaneous variation and direct mutant selection in cultures;
2. direct or indirect mutagenesis procedures on tissue culture of all cell types, seeds or plants; and
3. mutation of the cloned acetyl CoA carboxylase gene by methods such as site specific mutagenesis (Sambrook et al. cited supra), transposon mediated mutagenesis (Berg et al., Biotechnology, 1:417 (1983)) and deletion mutagenesis (Mitra et al., Molec. Gen. Genetic., 215:294 (1989)).

Mutants can be identified by a change in a functional activity of the enzyme encoded by the gene or by detecting a change in the DNA sequence using restriction enzyme mapping or partial sequence analysis.

In a preferred version, a functional mutant gene encoding for a plant acetyl CoA carboxylase tolerant to cyclohexanedione and/or aryloxyphenoxypropanoic acid herbicides is isolated from a maize herbicide tolerant cell line. The maize herbicide tolerant cell line was obtained as described in U.S. Patent No. 5,162,602, issued November 10, 1992, the disclosure of which is incorporated in Examples I-III. Briefly, partially differentiated cell cultures are grown and subcultured with continuous exposures to low herbicide levels. Herbicide concentrations are then gradually increased over several subculture intervals. Maize cells or tissues growing in the presence of normally toxic herbicide levels are repeatedly subcultured in the presence of the herbicide and characterized. Stability of the herbicide tolerance trait of the cultured cells may be evaluated by growing the selected cell lines in the absence of herbicides for various periods of time and then analyzing growth after exposing the tissue to herbicide.

Maize cell lines which are tolerant by virtue of having an altered acetyl CoA carboxylase enzyme can be selected by identifying cell lines having

enzyme activity in the presence of normally toxic levels of sethoxydim or haloxyfop. The tolerant maize cells can be further evaluated for whether acetyl CoA carboxylase is altered to a less sensitive form or increased in its level of expression.

Maize cell lines with a acetyl CoA carboxylase less sensitive to herbicide inhibition can be used to isolate a functional mutant gene of a plant acetyl CoA carboxylase. A DNA library from a maize cell line tolerant to herbicides can be generated and DNA fragments encoding all or a portion of an acetyl CoA carboxylase gene can be identified by hybridization to a cDNA probe encoding a portion of the maize ACCase gene. A complete copy of the altered gene can be obtained either by cloning and ligation or by PCR synthesis using appropriate primers. The isolation of the altered gene coding for acetyl CoA carboxylase can be confirmed in transformed plant cells by determining whether the acetyl CoA carboxylase being expressed retains enzyme activity when exposed to normally toxic levels of herbicides.

2. Promoters

Once a plant acetyl CoA carboxylase gene or functional mutant thereof or an antisense DNA sequence is obtained and amplified, it is combined with a promoter functional in a plant cell to form an expression cassette.

Most genes have regions of DNA sequence that are known as promoters and which regulate gene expression. Promoter regions are typically found in the flanking DNA sequence upstream from the coding sequence in both procaryotic and eukaryotic cells. A promoter sequence provides for regulation of transcription of the downstream gene sequence and typically includes from about 50 to about 2,000 nucleotide base pairs. Promoter sequences also contain regulatory sequences such as enhancer sequences that can influence the level of gene expression. Some isolated promoter sequences can provide for gene expression of heterologous genes, that is a gene different from the native or homologous gene. Promoter sequences are also known to be strong or weak or inducible. A strong promoter provides for a high level of gene expression, whereas a weak promoter provides for a very low level of gene expression. An inducible promoter is a promoter that provides for turning on and off of gene expression in response to an exogenously added agent or to an environmental or

developmental stimulus. Promoters can also provide for tissue specific or developmental regulation. An isolated promoter sequence that is a strong promoter for heterologous genes is advantageous because it provides for a sufficient level of gene expression to allow for easy detection and selection of transformed cells and provides for a high level of gene expression when desired.

The promoter in an expression cassette of the invention can provide for overexpression of acetyl CoA of a plant acetyl CoA carboxylase gene or functional mutant thereof. Overexpression of the gene is that amount of gene expression that results in an increase in tolerance of the plant cells to an herbicide or that results in an increase in the total oil content of the cells. Overexpression of an acetyl CoA carboxylase gene is preferably about a 2- to 20-fold increase in expression of an acetyl CoA carboxylase over the expression level of the native acetyl CoA carboxylase. The promoter can also be inducible so that gene expression can be turned on or off by an exogenously added agent. For example, a bacterial promoter such as the P_{tac} promoter can be induced to varying levels of gene expression depending on the level of isothiopropylgalactoside added to the transformed bacterial cells. It may also be preferable to combine the gene with a promoter that provides tissue specific expression or developmentally regulated gene expression in plants.

Specific promoters functional in plant cells include the 35S cauliflower mosaic virus promoter, nopaline synthase (NOS) promoter and the like. Currently, a preferred promoter for expression in monocots is the 35S cauliflower mosaic virus promoter.

An acetyl CoA carboxylase gene can be combined with the promoter by standard methods as described in Sambrook cited supra. Briefly, a plasmid containing a promoter such as the 35S cauliflower mosaic virus promoter can be constructed as described in Jefferson, Plant Molecular Biology Reporter, 5,387 (1987) or obtained from Clontech Lab in Palo Alto, CA (e.g. pBI121 or pBI221). Typically these plasmids are constructed to provide for multiple cloning sites having specificity for different restriction enzymes downstream from the promoter. A gene for plant acetyl CoA carboxylase can be subcloned downstream from the promoter using restriction enzymes to ensure that the gene is inserted in proper orientation with respect to the promoter so that

the gene can be expressed. In a preferred version, a maize acetyl CoA carboxylase is operably linked to a 35 S Came promoter in a plasmid such as pBI121 or pBI221. Once a plant acetyl CoA carboxylase gene is operably linked to a promoter and the plasmid, the expression cassette so formed can be subcloned into other plasmids or vectors.

3. **Optional Sequences in the Expression Cassette**

The expression cassette can also optionally contain other DNA sequences. The expression cassette can further be comprised of a chloroplast transit peptide sequence operably linked between a promoter and a plant acetyl CoA carboxylase gene. If the expression cassette is to be introduced into a plant cell, the expression cassette can also contain plant transcriptional termination and polyadenylation signals and translational signals linked to the 3′ terminus of a plant acetyl CoA carboxylase gene. The expression cassette can also optionally be further comprised of a plasmid.

Because one site of action for biosynthetic pathways involving plant acetyl CoA carboxylase is the chloroplast, an expression cassette of the invention can be combined with an exogenous DNA sequence coding for a chloroplast transit peptide, if necessary. An exogenous chloroplast transit peptide is one which is not encoded within the plant acetyl CoA carboxylase gene. A chloroplast transit peptide is typically 40 to 70 amino acids in length and functions post-translationally to direct the protein to the chloroplast. The transit peptide is cleaved either during or just after import into the chloroplast to yield the mature protein. The complete copy of a gene encoding a plant acetyl CoA carboxylase may contain a chloroplast transit peptide sequence. In that case, it may not be necessary to combine an exogenously obtained chloroplast transit peptide sequence into the expression cassette.

Exogenous chloroplast transit peptide encoding sequences can be obtained from a variety of plant nuclear genes, so long as the products of the genes are expressed as preproteins comprising an amino terminal transit peptide and transported into chloroplast. Examples of plant gene products known to include such transit peptide sequences are the small subunit of ribulose biphosphate carboxylase, ferredoxin, chlorophyll a/b binding protein, chloroplast ribosomal proteins encoded by nuclear genes, certain heatshock proteins, amino

acid biosynthetic enzymes such as acetohydroxy acid synthase, 3-enolpyruvylphosphoshikimate synthase, dihydrodipicolinate synthase, and the like. Alternatively, the DNA fragment coding for the transit peptide may be chemically synthesized either wholly or in part from the known sequences of transit peptides such as those listed above.

Regardless of the source of the DNA fragment coding for the transit peptide, it should include a translation initiation codon and an amino acid sequence that is recognized by and will function properly in chloroplasts of the host plant. Attention should also be given to the amino acid sequence at the junction between the transit peptide and the plant acetyl CoA carboxylase enzyme where it is cleaved to yield the mature enzyme. Certain conserved amino acid sequences have been identified and may serve as a guideline. Precise fusion of the transit peptide coding sequence with the acetyl CoA carboxylase coding sequence may require manipulation of one or both DNA sequences to introduce, for example, a convenient restriction site. This may be accomplished by methods including site directed mutagenesis, insertion of chemically synthesized oligonucleotide linkers and the like.

Once obtained, the chloroplast transit peptide sequence can be appropriately linked to the promoter and a plant acetyl CoA carboxylase gene in an expression cassette using standard methods. Briefly, a plasmid containing a promoter functional in plant cells and having multiple cloning sites downstream can be constructed as described in Jefferson cited supra. The chloroplast transit peptide sequence can be inserted downstream from the promoter using restriction enzymes. A plant acetyl CoA carboxylase gene can then be inserted immediately downstream from and in frame with the 3′ terminus of the chloroplast transit peptide sequence so that the chloroplast transit peptide is linked to the amino terminus of the plant acetyl CoA carboxylase. Once formed, the expression cassette can be subcloned into other plasmids or vectors.

When the expression cassette is to be introduced into a plant cell, the expression cassette can also optionally include 3′ nontranslated plant regulatory DNA sequences. The 3′ nontranslated regulatory DNA sequence preferably includes from about 300 to 1,000 nucleotide base pairs and contains plant transcriptional and translational termination sequence. Specific examples

of 3′ nontranslated regulatory DNA sequences functional in plant cells include about 500 base pairs of the 3′ flanking DNA sequence of the pea ribulose biphosphate carboxylase small subunit E9 gene, the 3′ flanking DNA sequence of the octopine synthase gene, and the 3′ flanking DNA sequence of the nopaline synthase gene. These 3′ nontranslated regulatory sequences can be obtained as described in An, Methods in Enzymology, 153:292 (1987) or are already present in plasmids available from commercial sources such as Clontech, Palo Alto, CA. The 3′ nontranslated regulatory sequences can be operably linked to the 3′ terminus of a plant acetyl CoA carboxylase gene by standard methods.

An expression cassette of the invention can also be further comprised of a plasmid. Plasmid vectors included additional DNA sequences that provide for easy selection, amplification and transformation of the expression cassette in procaryotic and eukaryotic cells. The additional DNA sequences include origins of replication to provide for autonomous replication of the vector, selectable marker genes, preferably encoding antibiotic resistance, unique multiple cloning sites providing for multiple sites to insert DNA sequences or genes encoded in the expression cassette, and sequences that enhance transformation of prokaryotic and eukaryotic cells. The preferred vectors of the invention are plasmid vectors. The especially preferred vector is the pBI121 or pBI221 vector formed as described by Jefferson cited supra.

Another vector that is useful for expression in both plant and procaryotic cells is the binary Ti vector PGA582. This binary Ti vector has been previously characterized by An, cited supra., and is available from Dr. An. This binary Ti vector can be replicated in procaryotic bacteria such as E. coli and Agrobacterium. The Agrobacterium plasmid vectors can be used to transfer the expression cassette to plant cells. The binary Ti vectors preferably include the nopaline T DNA right and left borders to provide for efficient plant cell transformation, a selectable marker gene, unique multiple cloning sites in the T border regions, the colE1 replication of origin and a wide host range replicon. The binary Ti vectors carrying an expression cassette of the invention can be used to transform both prokaryotic and eukaryotic cells, but is preferably used to transform plant cells.

B. Method for Screening for Expression and/or Overexpression of a Plant Acetyl CoA Carboxylase Gene

A method for screening for expression or overexpression of a plant acetyl CoA carboxylase gene is also provided by the invention. Once formed, an expression cassette comprising an acetyl CoA carboxylase gene can be subcloned into a known expression vector. The screening method in the invention includes the steps of introducing an expression vector into a host cell and detecting and/or quantitating expression of a plant acetyl CoA carboxylase gene. This method of screening is useful to identify expression cassettes providing for an overexpression of a plant acetyl CoA carboxylase gene, antisense molecules that effectively inhibit acetyl CoA carboxylase synthesis, and expression of an acetyl CoA carboxylase in the chloroplast of a transformed plant cell.

Suitable known expression vectors include plasmids that autonomously replicate in prokaryotic and eukaryotic cells. Specific examples include plasmids such as the pBI121 or pBI221 plasmid constructed as described by Jefferson cited supra, a binary Ti vector such as PG582 as described by An cited supra, PUC119, or PBR322. The preferred expression system is a pBI121 or pBI221 plasmid.

An expression cassette of the invention can be subcloned into an expression vector by standard methods. The expression vector can then be introduced into prokaryotic or eukaryotic cells by standard methods including protoplast transformation, silicon fiber-based transformation Agrobacterium mediated transformation, electroporation, microprojectiles, e.g., preferably using gold particles of about 6 to about 10 microns in diameter although diameters that are about 100 to about 1000 times smaller may also be employed, and liposomes. The expression vector can be introduced into plant cells such as tobacco, Brassica, Black Mexican sweet corn, and Arabidopsis cells. The vector can also be introduced into procaryotic cells such as E. coli or Agrobacterium. Transformed cells can be selected typically using a selection marker encoded on the expression vector.

Transient expression of a plant acetyl CoA carboxylase gene can be detected and quantitated in the transformed cells. Gene expression can be

quantitated by a quantitative Western blot using antibodies specific for the cloned acetyl CoA carboxylase or by detecting an increase in specific activity of the enzyme. The tissue and subcellular location of the cloned acetyl CoA carboxylase can be determined by immunochemical staining methods using antibodies specific for the cloned acetyl CoA carboxylase. Sensitivity of the cloned acetyl CoA carboxylase to herbicides can also be assessed. Expression cassettes providing for overexpression of a plant acetyl CoA carboxylase or acetyl CoA carboxylase tolerant to herbicides can then be used to transform monocot and/or dicot plant tissue cells and to regenerate transformed plants and seeds.

C. Method of Imparting Cyclohexanedione or Aryloxyphenoxypropanoic Acid Herbicide Tolerance to a Plant

The invention provides a method of conferring cyclohexanedione or aryloxyphenoxypropanoic acid herbicide tolerance to a plant. The method includes the steps of introducing an expression cassette comprising a gene coding for a plant acetyl CoA carboxylase or a functional mutant thereof operably linked to a promoter into the cells of plant tissue and expressing the gene in an amount effective to render the cells of the plant tissue substantially tolerant to herbicides. An effective amount of gene expression to render the cells of the plant tissue substantially tolerant to the herbicide depends on whether the gene codes for an unaltered acetyl CoA carboxylase gene or a mutant or altered form of the gene that is less sensitive to the herbicides. Expression of an unaltered plant acetyl CoA carboxylase gene in an effective amount is that amount that provides for a 2- to 50-fold increase in herbicide tolerance and preferably increases the amount of acetyl CoA carboxylase from at least about 2- to 20-fold over that amount of the native enzyme. An altered form of the enzyme can be expressed at levels comparable to that of the native enzyme or less if the altered form of the enzyme has higher specific activity. Acetyl CoA carboxylase substantially tolerant to herbicides is an enzyme that is tolerant of levels of herbicide which normally inhibit a native acetyl CoA carboxylase and preferably can function in concentrations of herbicide of about 2- to 20-fold greater than are toxic to the native enzyme.

Herbicide tolerance can be achieved by at least two methods including: 1) by increasing the level of gene expression of a native or unaltered acetyl CoA carboxylase gene; or 2) by introducing an altered gene coding for an acetyl CoA carboxylase that is less sensitive to herbicide inhibition. The level of gene expression can be increased by either combining a plant acetyl CoA carboxylase gene with a promoter that provides for a high level of gene expression such as the 35S Came promoter or by introducing the gene into the cells so that multiple copies of the gene are integrated into the genome of the transformed plant cell. Formation of an expression cassette comprised of a plant acetyl CoA carboxylase gene operably linked to a promoter that can be expressed in an effective amount to confer herbicide tolerance has been described previously.

Most monocots, but not dicots, are sensitive to cyclohexanedione and/or aryloxyphenoxypropanoic acid herbicides. The preferred plant cells for introducing an expression cassette of the invention to achieve herbicide tolerance for the plant cells then are monocot plants. Monocot plants include corn, wheat, barley, sorghum, rice, and others. An expression cassette of the invention can be introduced by methods of transformation, especially effective for monocots including biolistic transformation of Type II embryogenic suspension cells as described by W.J. Gordon-Kamm et al., Plant Cell, 2, 603-618 (1990), M.E. Fromm et al., Bio/Technology, 8, 833-839 (1990) and D.A. Walters et al., Plant Molecular Biology, 18, 189-200 (1992) or by electroporation of type 1 embryogenic calluses described by D'Hafluin et al., The Plant Cell, 4, 1495 (1992). Transformed cells can be selected for the presence of a selectable marker gene. Transient expression of a plant acetyl CoA carboxylase gene can be detected in the transgenic embryogenic calli using antibodies specific for the cloned plant acetyl CoA carboxylase. Transformed embryogenic calli can be used to generate transgenic plants that exhibit stable inheritance of either the altered acetyl CoA carboxylase gene or overexpression of the acetyl CoA carboxylase gene. Maize cell lines exhibiting satisfactory levels of tolerance to herbicide are put through a plant regeneration protocol to obtain mature maize plants and seeds expressing the tolerance traits such as described in D'Hafluin, cited supra., or An, cited supra. The plant regeneration protocol allows the

development of somatic embryos and the subsequent growth of roots and shoots. To determine that the herbicide-tolerance trait is expressed in differentiated organs of the plant, and not solely in undifferentiated cell culture, regenerated plants are exposed to herbicide levels which will normally inhibit shoot and root formation and growth.

Mature maize plants are then obtained from maize cell lines that are known to express the trait. If possible, the regenerated plants are self-pollinated. Otherwise, pollen obtained from the regenerated plants is crossed to seed-grown plants of agronomically important inbred lines. Conversely, pollen from plants of these inbred lines is used to pollinate regenerated plants. The genetics of the trait are then characterized by evaluating the segregation of the trait in the first and later generation progeny. Stable inheritance of overexpression of a plant acetyl CoA carboxylase or a functional mutant of a plant acetyl CoA carboxylase conferring herbicide tolerance to the plant is achieved if the plants maintain herbicide tolerance for at least about three to six generations.

Seed from transformed monocot plants regenerated from transformed tissue cultures is grown in the field and self-pollinated to generate true breeding plants. Progenies from these plants become true breeding lines which are evaluated for herbicide tolerance in the field under a range of environmental conditions. Herbicide tolerance must be sufficient to protect the monocot plants at the maximum labeled delivery rate under field conditions which cause herbicides to be most active. Appropriate herbicide concentrations and methods of application are those which are known and have been developed for the cyclohexanedione and/or aryloxyphenoxypropanoic acid herbicides in question.

In a preferred version, an expression cassette comprised of a maize acetyl CoA carboxylase gene isolated from a maize cell line tolerant to sethoxydim and haloxyfop and linked to the 35S Came promoter is introduced into an herbicide sensitive monocot tissue using biolistic transformation. Transformed calli are selected and used to generate transgenic plants. Transformed calli and transgenic plants can be evaluated for tolerance to sethoxydim and haloxyfop and for stable inheritance of the tolerance trait.

D. Method for Altering the Oil Content in a Plant

The invention also provides a method of altering the oil content in a plant. The method include the steps of introducing an expression cassette comprising a gene coding for plant acetyl CoA carboxylase or functional mutant thereof operably linked to a promoter functional in a plant cell into the cells of plant tissue and expressing the gene in an amount effective to alter the oil content of the plant cell. An alteration in the oil content of a plant cell can include a change in the total oil content over that normally present in that type of plant cell, or a change in the type of oil from that normally present in the plant cell. Expression of the gene in an amount effective to alter the oil content of the gene depends on whether the gene codes for an unaltered acetyl CoA carboxylase or a mutant or altered form of the gene. Expression of an unaltered plant acetyl CoA carboxylase gene in an effective amount is that amount that may provide a change in the oil content of the cell from about 1.2- to 20-fold over that normally present in that plant cell, and preferably increases the amount of acetyl CoA carboxylase about 2- to 20-fold over that amount of the enzyme normally present in that plant cell. An altered form of the enzyme can be expressed at levels comparable to that of the native enzyme or less if the altered form of the enzyme has higher specific activity.

An alteration in oil content of the plant cells according to the method of the invention can be achieved in at least two ways including:

(1) an increase or decrease in expression of an unaltered plant acetyl CoA carboxylase gene; or

(2) by introducing an altered or functional mutant plant acetyl CoA carboxylase gene coding for an enzyme that exhibits a change in specific activity.

The level of gene expression of an unaltered plant acetyl CoA carboxylase gene can be increased by either combining an unaltered plant acetyl CoA carboxylase gene with a promoter that provides for a high level of gene expression, such as the 35S cauliflower mosaic virus or by introducing the expression cassette and/or selecting for plant cells having multiple copies of a plant acetyl CoA carboxylase gene integrated into the genome. A decrease in expression of an unaltered acetyl CoA carboxylase can be achieved by transformation with an ACCase antisense

gene containing an expression cassette. When an altered or functional mutant plant acetyl CoA carboxylase gene codes for an enzyme that has an increase in specific activity, it may lead to an increase in total oil content of a plant cell even if the level of gene expression is comparable to that of the native enzyme. When an altered or functional mutant acetyl CoA carboxylase gene codes for an enzyme having a decrease in specific activity, it may lead to a decrease in the total oil content of the plant cell compared to that normally present.

An expression cassette as described above can be introduced into either monocots or dicots. Preferably, the expression cassette is introduced into dicot plants such as soybean, canola, and sunflower. An expression cassette can be introduced by standard methods including protoplast transformation, Agrobacterium-mediated transformation, microprojectiles, electroporation, and the like. Transformed cells or tissues can be selected for the presence of a selectable marker gene.

Transient expression of a plant acetyl CoA carboxylase gene can be detected in transformed cells or tissues by immunoreactivity with antibodies specific for the cloned acetyl CoA carboxylase. Overexpression of a plant acetyl CoA carboxylase can be detected by quantitative Western blots. A change in specific activity of the enzyme can be detected by measuring enzyme activity in the transformed cells. A change in total oil content can also be examined by standard methods, as described in Clark & Snyder, JACS, 66:1316 (1989).

Transgenic plants and seeds can be generated from transformed cells and tissues showing a change in oil content or in the amount or specific activity of a plant acetyl CoA carboxylase using standard methods. It is especially preferred that the oil content of the leaves, seeds, or fruits is increased.

In a preferred version, a maize acetyl CoA carboxylase gene is combined with a 35S cauliflower mosaic virus promoter in a vector such as pBI121 or pBI221 and introduced into soybean cells using the microprojectile method. Transformed soybean cells showing an increase in expression of acetyl CoA carboxylase of at least about 2-fold or at least a 1.2-fold increase in oil content are selected. Transformed soybean cells exhibiting overexpression of acetyl CoA carboxylase or showing an increase in total oil content are used to generate transgenic plants and seeds.

E. Method of Producing Plant Acetyl CoA Carboxylase

The invention also provides a method of producing plant acetyl CoA carboxylase in a host cell. The method includes the steps of introducing an expression cassette comprised of a gene encoding a plant acetyl CoA carboxylase or functional mutant thereof into a host cell and expressing the gene in an amount sufficient to allow for crystallization of the plant acetyl CoA carboxylase. An amount sufficient to allow for crystallization of a plant acetyl CoA carboxylase is about 20- to 100-fold increase over the amount of plant acetyl CoA carboxylase that can normally be purified from plant cells, preferably about 2 to 10 mg protein. Crystallized plant acetyl CoA carboxylase can be used to identify other herbicides that can bind to and inhibit acetyl CoA carboxylase function. In addition, the availability of large amounts of purified enzyme provides for screening of the efficacy of such herbicides.

An expression cassette can include a promoter that is functional in either a eukaryotic or prokaryotic cell. The expression cassette can be introduced into a prokaryotic cell such as E. coli, or a eukaryotic cell such as a plant or yeast. The preferred cell is a prokaryotic cell used routinely in producing recombinant proteins such as E. coli. The expression cassette can be introduced and transformed cells selected by standard methods.

The plant acetyl CoA carboxylase gene can be expressed in an prokaryotic cell until sufficient amount of the enzyme is produced so that it can be crystallized. Plant acetyl CoA carboxylase can be isolated from bacterial cells using standard methods, including those described in Example V. The purified acetyl CoA carboxylase can then be crystallized and characterized by standard methods.

EXAMPLE I

Identification of Herbicide Mechanism and Site of Action

The objective of this Example was to identify the mechanism whereby sethoxydim and/or haloxyfop inhibit fatty acid synthesis in maize. The results, reported in J. D. Burton et al., Biochem. Biophys. Res. Comm., 148, 1039 (November 13, 1987), show that both sethoxydim and haloxyfop inhibit acetyl-coenzyme A carboxylase (ACCase) (EC 6.4.1.2) in maize chloroplasts.

A. Chemicals

Buffers and cofactors were purchased from Sigma Chemical Company (St. Louis, Missouri); [2-^{14}C]acetate was purchased from Research Products International; [2-^{14}C]pyruvate and [^{14}C]$NaHCO_3$ were purchased from New England Nuclear; and [2-^{14}C]malonyl coenzyme A was purchased from Amersham. Sethoxydim was a gift from BASF (Parsippany, New Jersey), and haloxyfop was provided by Dow Chemical USA (Midland, Michigan).

B. Plant Growth Conditions

Corn (Z. mays L., 'B37 x Oh43') seeds were germinated in darkness for 96 hours in vermiculite in an incubation chamber maintained at 30°C, 80% RH. Seedlings were then transferred to a growth chamber with a 16 hour light (25°C) and an 8 hour dark (20°C) cycle, 90% relative humidity (RH). After greening 48 hours, seedlings were returned to the dark incubation chamber for 12 hours to deplete chloroplast starch reserves. Seedlings were harvested 6 days after planting. Pea (P. sativum L., 'PI 9901-C') seedlings were grown in vermiculite in a growth chamber with a 16 hour light (21°C) and 8 hour dark (16°C) cycle, 80% RH. Peas were harvested 10 to 13 days after planting. Black Mexican Sweet (BMS) corn suspension cultures were maintained in a supplemented Murashige-Skoog (MS) medium (C. E. Green, Hort. Sci., 12, 7-10 (1977)), and subcultured weekly by 20-fold dilution of the suspension culture into fresh medium.

C. Chloroplast Isolation

Chloroplasts from corn and pea seedlings were isolated at 4°C (K. Cline et al., J. Biol. Chem., 260, 3691-3696 (1985)). Seedlings (50 g of shoots) were homogenized in 200 ml buffer A (50 mM HEPES-NaOH pH 7.5, 330 mM sorbitol, 0.1% w/v BSA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 5 mM isoascorbate, 1.3 mM glutathione) in an omnimixer (five, 3-second bursts at full speed). The homogenate was filtered through six layers of cheesecloth and two layers of miracloth, and then centrifuged at 3000 g for 3 minutes with hand-braking. The pellet was gently resuspended in buffer A and layered onto a preformed linear Percoll gradient (50 mM HEPES-NaOH pH 7.5, 330 mM sorbitol, 1.9 mM isoascorbate, 1.08 mM glutathione, 0.1% w/v BSA, 50% Percoll) which was centrifuged at 3000 g for 20 minutes in a Sorvall HB-4 rotor.

The lower band in the gradient, containing intact chloroplasts, was washed twice by gently resuspending it in 20 ml of buffer B (50 mM HEPES-NaOH, pH 7.5, and 330 mM sorbitol) followed by repelleting (3000 g, 5 minutes). The final pellet, consisting of intact chloroplasts, was resuspended in 2 to 3 ml of buffer B and stored on ice in the dark until use.

D. Fatty Acid Synthesis

[^{14}C]acetate and [^{14}C]pyruvate were used as precursors to measure fatty acid biosynthesis in isolated chloroplasts (B. Liedvogel et al., Planta, 169, 481-489 (1986)). [^{14}C]acetate incorporation was assayed in a 0.5 ml-volume containing: 50 mM HEPES-NaOH (pH 7.5), 330 mM sorbitol, 5 mM KH_2PO_4, 10 mM $NaHCO_3$, 1 mM $MgCl_2$, 1 mM ATP, 0.1 mM CoA, 0.15 mM [^{14}C]acetate (3.33 mCi/mmol), and chloroplasts (20 to 50 µg chlorophyll). [^{14}C]pyruvate incorporation into fatty acids was assayed in the same medium except that it included 2 mM TPP, 1 mM NAD^+, 0.15 mM [^{14}C]-pyruvate (1.33 mCi/mmol), but no acetate. Assay suspensions were illuminated with 1400 µE/m^2·second PAR at 25°C. Assays were initiated by the addition of the labeled substrate and stopped by the addition of 0.5 ml of 40% KOH. To determine the incorporation of radiolabel into a non-polar (fatty acid) fraction, each treatment was saponified at 90°C for 30 minutes in capped vials (P. B. Hoj et al., Carlsberg Res. Commun., 47, 119-141 (1982)). The vials were acidified with 0.5 ml 40% H_2SO_4, and carrier fatty acids (20 µg each of C 14:0, C 16:0, and C 18:0) were added. The assay mixture was extracted twice with 4 ml hexane. The extracts were combined, dried under N_2, and redissolved in 0.3 ml hexane. Aliquots (50 µl) were counted for radioactivity by liquid scintillation spectrometry.

Incorporation of [^{14}C]malonyl-Coenzyme A into fatty acids (P. B. Hoj et al., supra; and J. B. Ohlrogge et al., Proc. Natl. Acad. Sci. USA, 76, 1194-1198 (1979)) was assayed using cell-free preparations from BMS tissue culture. Cells harvested during logarithmic growth phase were frozen in liquid nitrogen, ground with a mortar and pestle, and thawed in a medium containing: 0.1 M HEPES-KOH, pH 7.5; 0.3 M glycerol, and 5 mM DTT (buffer:tissue, 2:1, v/w). The homogenate was centrifuged at 12,000 g for 20 minutes. The supernatant was filtered through miracloth and centrifuged (125,000 g) for 60 minutes and then filtered through miracloth and assayed. Assays were conducted at 25°C in a

0.4 ml volume containing: 1.0 mM ATP, 0.32 mM NADPH, 0.38 mM NADH, 25 μM CoA, 10 μM acetyl-CoA, 25 μg acyl-carrier protein, and 12 μM malonyl-CoA (11.54 μCi/μmol). Reactions were initiated by addition of [^{14}C]malonyl CoA and stopped by addition of 0.4 ml 40% KOH. Label incorporation into fatty acids was determined as above. Chlorophyll (D. I. Arnon, Plant Physiol., 24, 1-15 (1949)) and protein (P. K. Smith et al., Anal. Biochem., 150, 76-85 (1985)) were determined as described therein.

E. Acetyl-Coenzyme A Carboxylase (ACCase) Activity

Maize chloroplasts, isolated as described above, were suspended in buffer C (0.1 M Tricine-KOH, pH 8.0; 0.3 M glycerol, and 1 mM DTT) and homogenized in a glass tissue homogenizer. The disrupted chloroplast fraction was centrifuged at 16,000 g for 15 minutes. The supernatant was desalted on a Sephadex G-25 column (1.5 x 5 cm equilibrated with 0.1 M Tricine-KOH, pH 8.0; and 0.3 M glycerol) and assayed directly. ACCase activity (B. J. Nikolau et al., Arch. Biochem. Biophys., 211, 605-612 (1981)) was assayed at 30°C in a 0.2 ml volume which contained 1 mM ATP, 3 mM acetyl coenzyme A, 2.5 mM $MgCl_2$, 50 mM KCl, 0.5 mM DTT, and 15 mM [^{14}C]$NaHCO_3$ (0.17 mCi/mmol). Reactions were initiated by addition of acetyl coenzyme A and stopped by addition of 25 μl of 12 N HCl. Product formation was determined by the radioactivity found in an acid stable fraction by liquid scintillation spectrometry. Enzyme activity was linear for 15 minutes.

F. Results

To probe for the site of herbicidal activity of sethoxydim and haloxyfop, labeled acetate, pyruvate, and malonyl-CoA were used individually as precursors for fatty acid synthesis. Isolated chloroplasts from corn seedlings incorporated [^{14}C]acetate and [^{14}C]pyruvate into a non-polar fraction (fatty acids). Acetate incorporation was linear for 30 min after a 5 min lag period, and dependent upon the addition of free acetyl coenzyme A. Addition of either 10 μM sethoxydim or 1 μM haloxyfop inhibited [^{14}C]acetate incorporation into fatty acids by 90% and 89%, respectively, as shown in Table I, below. Sethoxydim (10 μM) and haloxyfop (1 μM) also inhibited the incorporation of [^{14}C]pyruvate into fatty acids by 98% and 99%, respectively.

TABLE I

Inhibition of [^{14}C]acetate and [^{14}C]pyruvate Incorporation into Fatty Acids in Corn Seedling Chloroplasts by Sethoxydim (10 μM) and Haloxyfop (1 μM), 10 minute assay time

	Acetate	Pyruvate
	---Activity (nmol/mg chl·min)---	
Control	4.4 ± 0.4[1]	10.8 ± 2.3
	----------% Inhibition----------	
Sethoxydim	90 ± 2.5	98 ± 1.1
Haloxyfop	89 ± 3.1	99 ± 0.3

[1]Results are expressed as mean of two experiments ± standard error.

The effect of 10 μM sethoxydim and 1 μM haloxyfop on [^{14}C]malonyl-CoA incorporation into fatty acids was determined using cell-free extracts from corn suspension cultures. Neither sethoxydim (10 μM) nor haloxyfop (1 μM) inhibited fatty acid synthetase activity. Thus, both herbicides inhibited fatty acid synthesis in intact chloroplasts from corn seedlings with either acetate or pyruvate as a precursor, but did not inhibit incorporation of malonyl-CoA into fatty acids. This suggests that ACCase which catalyzes the formation of malonyl-CoA is the site of action of these herbicides.

EXAMPLE II

Selection and Characterization of Herbicide-tolerant Cell Lines

A selection protocol to identify and isolate herbicide-tolerant maize cells was developed to minimize the adverse effects of high herbicide concentrations on somatic embryo development and plant regeneration capacity. The procedure involved exposing tissue to gradually increasing concentrations of herbicide beginning with a sethoxydim concentration representing 1/20th of lethal dose and doubling the herbicide concentration at approximately two-week intervals until the lethal dose (10 μM sethoxydim) was reached. In this way, the

herbicide was allowed to take effect slowly with continuous selection pressure, thus permitting herbicide-tolerant cells to accumulate over time while not affecting the potential for plant regeneration.

A. Selection of a Sethoxydim-Tolerant Cell Line

Many selections were carried out utilizing the selection protocol described in the preceding paragraph. The selection of one such sethoxydim-tolerant cell line that was identified and characterized is described below in detail.

Approximately 100 grams of vigorously growing, regenerable, friable, embryogenic maize callus tissue established from an F_1 immature embryo resulting from the cross A188 x B73 were transferred to agar-solidified maintenance medium (Armstrong and Green, Planta, 164, 207 (1985)) in petri plates containing 0.5 μM sethoxydim (BASF) (Parsippany, New Jersey). This callus line was designated 2167-9/2160-154. Forty plates were prepared and five clumps of callus tissue weighing about 0.5 grams each were placed on each plate. The 0.5 μM sethoxydim concentration was chosen from growth inhibition studies to provide less than 10-20% growth inhibition during the first two weeks of herbicide exposure. After 14 days, 0.25-0.5 gram pieces of tissue showing vigorous growth rate and retention of embryogenic morphology (i.e., presence of somatic embryos) were subcultured on fresh medium containing 1.0 μM sethoxydim. Eighty plates containing five pieces of tissue per plate were prepared. For each subsequent transfer, all callus tissue showing growth and somatic embryo forming ability was placed on fresh media containing a two-fold increased sethoxydim concentration. Therefore, callus was transferred at two-week intervals to petri plates containing 0.5, 1.0, 2.0, 5.0 and 10.0 μM sethoxydim. During the course of the selection process, the total number of lines decreased as the herbicide-mediated growth inhibition became more intense. Cell lines exhibiting growth on 10 μM sethoxydim were designated as herbicide-tolerant and given an identification number. Two sethoxydim-tolerant lines were recovered that exhibited uninhibited growth at 10 μM sethoxydim. These lines were designated 2167-9/2160-154 S-1 and 2167-9/2160-154 S-2.

B. **Characterization of Herbicide-Tolerant Maize Cell Line 2167-9/2160-154 S-2**

Tolerant cell line 2167-9/2160-154 S-2 ("S-2") was characterized to evaluate: (1) the magnitude of sethoxydim tolerance; (2) cross-tolerance of haloxyfop; and (3) the biochemical basis for the tolerance. Callus tissue from S-2 that had been maintained on 10 μM sethoxydim was transferred to media containing up to 100 μM sethoxydim. One-half gram of S-2 tissue was plated on a 7 cm filter paper as a lawn overlaying 50 ml agar-solidified culture medium containing 0, 0.5, 1.0, 2.0, 5.0, 10.0, 50.0 and 100 μM sethoxydim, and cultured for two weeks. Control cell line 2167-9/2160-154 was plated similarly on medium containing the same levels of sethoxydim. The results of this study are summarized in Figure 2. The control cell line growth after two weeks was inhibited 50% at 1 μM sethoxydim. Growth of S-2 was not inhibited at 100 μM sethoxydim, indicating that S-2 was at least 100-fold more tolerant than the control callus line.

Growth of S-2 was inhibited with 0.65 μM haloxyfop, whereas the control cell line was inhibited 50% with 0.02 μM, indicating approximately a 30-fold increase in tolerance.

C. **Acetyl-Coenzyme A Carboxylase (ACCase) Activity of Maize Cell Line S-2**

Assays were conducted to determine if ACCase extracted from cell line S-2 was altered with respect to herbicide activity. ACCase activity of control tissue was 50% inhibited either by 1.5 μM sethoxydim, or by 0.25 μM haloxyfop. ACCase activity of S-2 tissue was inhibited 50% either by 70 μM sethoxydim, or by 1.8 μM haloxyfop, indicating at least 40-fold and 7-fold decreases in herbicide sensitivity on concentration basis, respectively.

EXAMPLE III

Plant Regeneration and Production of Herbicide-Tolerant Seed

A. **Plant Regeneration Protocol**

Sixteen ca. 150 mg clumps of S-2 callus were transferred per 25 x 100 mm petri plate containing agar-solidified N6 basal salts and 6% sucrose and incubated 7-14 days in low light (20 $\mu E\ m^{-2}\ s^{-1}$). Several plates containing callus on plant regeneration medium were prepared. Callus was transferred to agar-

solidified Murashige-Skoog (MS) medium without hormones and incubated in high intensity light (200 μE m-2 s^{-1}) for shoot elongation. Developing plants (1-3 cm long) were isolated from the callus surface and transferred to magenta boxes containing agar-solidified MS salts, 2% sucrose with no hormones for two weeks of further growth. When plants reached the 2-3 leaf stage, they were transplanted to peat pots containing potting soil, and were incubated in the growth room until growing stably. Surviving plants were transferred to soil in 4" diameter plastic pots and grown in the greenhouse.

B. Expression of Herbicide Tolerance in Plants Regenerated from S-2 Callus Tissue

Groups of eight control (2167-9/2160-154 unselected) and eight S-2 plants were sprayed with either 0.0, 0.01, 0.05, 0.11, 0.22 or 0.44 kg/ha sethoxydim to determine whole plant sethoxydim-tolerance of greenhouse-grown plants. Control plants were killed by 0.05 kg/ha or more sethoxydim. Plants regenerated from the S-2 cell line survived the 0.44 kg/ha sethoxydim treatment, indicating that S-2 plants exhibit at least 20-fold more tolerance of sethoxydim than control. Figure 3 shows the growth response of the regenerated plants seven days after treatment with 0.44 kg/ha sethoxydim. As shown in Figure 4, shoot height of regenerated S-2 plants was only slightly reduced 14 days after treatment with 0.44 kg/ha sethoxydim.

C. Seed Production from S-2 Plants

Plants surviving sethoxydim treatments of up to 0.44 kg/ha were transplanted to the genetics plot on the University of Minnesota campus, St. Paul, Minnesota. Additional S-2 plants were transplanted to the field that had not been sprayed. Sixty-five 2167-9/2160-154 control plants and ninety-five S-2 plants were grown to maturity in the field. Plants were either self-pollinated or cross-pollinated to inbred maize lines A188, A619, A641, A661, A665, B37, B73, R806, and W153R. Control seed were produced by selfing 2167-9/2160-154 regenerated plants, or by crossing them with the inbreds listed above.

D. Expression of Herbicide Tolerance in Progeny of Regenerated Plants

Seeds obtained by the crossing procedure described above were viable and germinated normally. Seeds from thirty S-2 selfed plants and fifteen 2167-9/2160-154 control plants were planted in 25 x 50 cm trays of soil (28

seeds from each plant in one tray) and grown in the greenhouse. Seedlings at the 3-4 leaf stage were treated with 0.1, 0.44, and 1.1 kg/ha sethoxydim and evaluated for visual herbicide damage and shoot height. Based on visual rating of herbicide damage two weeks after treatment, selfed progeny of S-2 plants segregated approximately 1:2:1 for healthy, uninjured plants: to plants showing partial injury: to dead plants, respectively, at 0.44 and 1.1 kg/ha sethoxydim treatments. All control progeny of 2167-9/2160-154 control plants were killed by 0.1 kg/ha and greater levels of sethoxydim. These results demonstrate dominant expression of sethoxydim tolerance indicating that sethoxydim tolerance in S-2 plants is a heritable trait. Similar tests were conducted on progeny of S-2 plants crossed to the other inbreds. In all cases, these test cross progeny treated with 0.44 kg/ha sethoxydim segregated 1:1 for growing shoots versus dead shoots whether S-2 plants were used as male or female parents. These results confirm that sethoxydim tolerance is controlled by a single dominant nuclear gene. In all cases, control plants crossed to the other inbreds were killed and therefore sethoxydim-sensitive.

E. Method for Obtaining Uniform Herbicide-Tolerant Seed

Progeny of S-2 plants surviving sethoxydim treatments of 0.44 and 1.1 kg/ha and showing no herbicide injury were transferred to the greenhouse and grown to maturity. These plants may be selfed and their progeny evaluated for sethoxydim and haloxyfop tolerance to identify pure breeding herbicide-tolerant maize lines.

Progeny of S-2 plants crossed to inbred lines and exhibiting sethoxydim tolerance may be recurrently backcrossed to the same inbreds. Progeny of each cross may be screened for sethoxydim-tolerance, and tolerant plants grown to maturity and again crossed to the recurrent parent. After six or seven cycles of backcrossing, sethoxydim-tolerant plants may be selfed and progeny screened for tolerance to produce homozygous sethoxydim tolerant maize inbreds.

EXAMPLE IV

Selection of Additional Herbicide-Tolerant Maize Cell Lines

One primarily sethoxydim-tolerant maize cell line, 2167-9/2160-154 S-1, and two haloxyfop-tolerant maize cell lines, 2167-9/2160-154 H-1 and 2167-9/2160-154 H-2, were selected and characterized as follows:

A. Selection of Maize Cell Line 2167-9/2160-154 S-1

Maize cell line 2167-9/2160-154 S-1 was selected from maize cell culture using the protocol described in detail above for the selection of Line 2167-9/2160-154 S-2. Approximately 70 plants were regenerated from Line 2167-9/2160-154 S-1, and either self-pollinated or cross-pollinated to the inbred maize lines A188, A619, A641, A661, A665, B37, B73, R806, and W153R.

B. Selection of Maize Cell Line 2167-9/2160-154 H-1

Line 2167-9/2160-154 H-1 was selected from maize cell culture using a similar protocol described in detail above except maize callus tissue was selected using the herbicide haloxyfop. Maize callus tissue was initially plated on 0.01 μM haloxyfop. At two-week intervals, surviving tissue was subcultured onto 0.05, 0.10 and 0.20 μM haloxyfop. Approximately 50 plants were regenerated from Line 2167-9/2160-154 H-1, and were self-pollinated.

C. Selection of Maize Cell Line 2167-9/2160-154 H-2

Line 2167-9/2160-154 H-2 was selected from maize cell culture using a similar protocol described in detail for line 2167-9/2160-154 H-1. No plants have been successfully regenerated from this line.

D. Characterization of Lines 2167-9/2160-154 S-1, H-1 and H-2

The tolerant callus cultures were characterized to determine the magnitude of sethoxydim and haloxyfop tolerance. Callus tissue from these lines was evaluated in experiments as described above in the characterization of line 2167-9/2160-154 S-2. Table II summarizes the results of these studies. Line 2167-9/2160-154 S-1 and Line 2167-9/2160-154 H-2 showed a four-fold increase in haloxyfop tolerance, while Line 2167-9/2160-154 H-1 exhibited approximately a 60-fold increase in haloxyfop tolerance. Neither haloxyfop selected line showed a significant degree of sethoxydim tolerance, while the

sethoxydim selected line S-1 exhibited approximately a 100-fold increase in sethoxydim tolerance.

TABLE II

Herbicide Tolerance of Cell Lines S-1, H-1 and H-2

Cell Line	Herbicide	
	Haloxyfop	Sethoxydim
2167-9/2160-154 S-1	4[1]	100
2167-9/2160-154 H-1	61	0
2167-9/2160-154 H-2	4	0

[1]The numbers represent the fold increase in herbicide concentration that results in a 50% reduction in growth of the selected cell lines compared to the unselected control cell line 2167-9/2160-154.

E. Herbicide Inhibition of Acetyl Coenzyme A Carboxylase of Maize Cell Lines S-1, H-1 and H-2

Acetyl Coenzyme A Carboxylase (ACCase) was extracted from cell lines S-1, H-1 and H-2 and assayed as described in detail for maize cell line S-2, above. Table III below summarizes the results of these studies. The ACCase from line S-1 was more tolerant of both sethoxydim and haloxyfop, while the ACCase from line H-1 was more tolerant of haloxyfop, but not of sethoxydim. The ACCase from line H-2 showed no difference from the unselected parent line 2167-9/2160-154 in sensitivity to either herbicide.

However, cell line H-2 exhibited approximately a five-fold higher level of ACCase activity as compared to the unselected parent line 2167-9/2160-154. Thus, selection for sethoxydim or haloxyfop tolerance resulted in a less

sensitive ACCase in cell line S-1 and H-1, as well as a higher level of ACCase activity in cell line H-2.

TABLE III

Herbicide Inhibition of ACCase of Maize Cell Lines S-1, H-1 and H-2

Cell Line	Herbicide	
	Haloxyfop	Sethoxydim
2167-9/2160-154 S-1	3	4
2167-9/2160-154 H-1	7	0
2167-9/2160-154 H-2	0	0

[1]The numbers represent the fold increase in herbicide concentration that inhibits ACCase activity of the selected cell lines by 50% compared to the unselected parent cell line 2167-9/2160-154.

Deposit of Seeds

Seeds from representative S-2 plants (Ex. III (B)) and H-1 plants (Ex. IV(B)) have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852 USA on October 25, 1988 and assigned accession numbers ATCC 40507, and ATCC 40508, respectively.

EXAMPLE V

Formation of cDNA Clones Encoding ACCase

A. ACCase Purification

The acetyl CoA carboxylase enzyme was isolated and purified from plant tissues and characterized. The purified enzyme was used to generate antibody reagents useful in identifying cDNA clones encoding the gene or portions of the gene for ACCase.

ACCase was extracted from frozen shoots of 7-d-old maize (Zea Mays L. inbred A619 or B73) seedlings grown in a growth chamber (24°C, 90% RH, 16-h daylength at 210 $\mu E\ m^{-2}\ s^{-1}$). The outermost leaf and blade were removed and the remainder of the shoot was frozen in liquid N_2. Embryos and endosperm tissue from developing kernels were harvested from field-grown ears at 36 to 40 days after pollination (DAP). Black Mexican Sweet corn (BMS)

maize suspension cells were obtained from cultures as previously described (W.B. Parker et al., Plant Physiol., 92, 1220-1225 (1990)). Tissues were stored in liquid N_2 until used.

Extraction and purification steps were performed at 0 to 4°C. Crude extracts of leaf, bundle sheath strands, embryo, endosperm, and BMS cells were prepared from frozen tissue as described by W.B. Parker et al., Proc. Nat'l Acad. Sci. USA, 87, 7175-7179 (1990), except that extraction buffer contained 0.1 M Tricine-KOH, pH 8.3, 0.3 M glycerol, 5 mM DTT, 2mM Na_2EDTA, and 0.5 mM phenyl methonyl sulfonyl fluoride (PMSF). Triton X-100 (0.01% v/v) was added to bundle sheath strand extracts and to some whole leaf extracts. For some experiments, additional protease inhibitors (leupeptin, 2 μg mL; pepstatin A, 100 μg mL^{-1}; benzamidine, 1 mM; ε-amino-n-caproic acid, 5 mM; and soybean trypsin inhibitor, 10 μg mL^{-1}) were included. Filtered homogenates were centrifuged 20 minutes at 30,000g. A portion of the crude supernatant fraction was immediately boiled 5 minutes in 1 volume of SDS sample buffer (W.B. Parker et al., Plant Physiol., 92, 1220-1225 (1990)) for SDS-PAGE analysis; the remainder was desalted on a 10-mL Sephadex G-25 column into extraction buffer minus PMSF.

ACCase was purified from the crude extract supernatant in four steps. This fraction was brought to 30% saturation with solid $(NH_4)_2SO_4$, stirred 15 minutes, and centrifuged 20 minute at 20,000g. The supernatant was then brought to 40% saturation with $(NH_4)_2SO_4$ solution, stirred 30 minutes, and centrifuged. The pellet was dissolved in 5 mL extraction buffer, microfuged 5 minutes, and the resulting supernatant was applied to a Sephacryl S-300 gel filtration column (Pharmacia; 2.5 x 46 cm) equilibrated with S-300 buffer (0.1 M Tricine-KOH, pH 8.3, 0.5 M glycerol, 0.5 mM DTT, 2 mM Na50 mM KCl). In later experiments a Sephacryl S-400 column was used. Fractions (2.5 mL) were eluted at 0.75 mL min^{-1}. ACCase activity eluted shortly after the void A_{280} peak (V_0 = 75 mL). Active fractions were pooled, brought to 4.25 mM $MgCl_2$ (from a 0.5 M solution), and applied at 0.2 mL min^{-1} to a Blue Sepharose CL-6B (Pharmacia; 1.5 x 15 cm) equilibrated with Blue sepharose buffer (S-300 buffer containing 4.25 mM $MgCl_2$ and 10 mM $NaHCO_3$). The column was washed overnight with 150 mL buffer (0.45 mL min^{-1}). ACCase activity was then eluted

with 50 mL buffer plus 10 mM ATP (0.45 mL min^{-1}). Active fractions were pooled and applied to an FPLC Mono-Q HR 5/5 anion-exchange column (Pharmacia) equilibrated with S-300 buffer minus KCl. The column was washed with 30 mL S-300 buffer minus KCl and then with a 48-mL, 0 to 500 mM KCl gradient in S-300 buffer (0.25 mL min^{-1}). Fractions (1 mL) from the two peaks of ACCase activity were pooled separately. All purification fractions were desalted into S-300 buffer and assayed for ACCase activity and protein.

ACCase was also analyzed from mesophyll chloroplasts and bundle sheath strands. Mesophyll chloroplasts from homogenates of 7- to 8-day-old seedlings that were kept in the dark 24 hours prior to harvesting were isolated on a linear Percoll gradient according to J.D. Burton et al., Pesticide Biochemistry and Physiology, 34, 76-85 (1989), except that buffers contained 0.6 M sorbitol and centrifugation *g*-forces were reduced by 25%. Intact chloroplasts were taken from the discrete lower green band present after Percoll gradient centrifugation (G. Morioux et al., Plant Physiol., 67, 470-473 (1981)). Pelleted chloroplasts were lysed by resuspending them in ACCase extraction buffer plus PMSF and 0.01% (v/v) Triton X-100. Bundle sheath strands were obtained from the original leaf homogenate material retained on a 70-μm filter after re-homogenizing the retentate five times in a total of 2 L buffer. Triton X-treated, desalted leaf, mesophyll chloroplast, and bundle sheath strand extracts were assayed for activities of Rubisco (G. Zhu et al., Plant Physiol., 97, 1348-1353 (1991)), NADP-dependent malate dehydrogenase (M.D. Hatch et al., Biochem. Biophys. Res. Commun., 34, 589-593 (1969)), phosphoenolpyruvate carboxylase (R.C. Leegood et al., "Isolation of Membranes and Organelles from Plant Cells," Academic Press, New York, 185-210(1983)), catalase (Worthington Biochemicals, 1972), and fumarase (R.L. Hill et al., Methods Enzymol., 13, 91-99 (1969)), and for total chlorophyll (D.E. Arnon, Plant Physiol., 24, 1-5 (1949)). Mesophyll chloroplast preparations were judged to be relatively free of contamination by bundle sheath chloroplasts because they contained 3-fold greater NADP-dependent malate dehydrogenase and one-tenth as much Rubisco activity (mg^{-1} chlorophyll) than bundle sheath strand extracts. Mesophyll chloroplast preparations also contained ≤2.6% as much catalase, fumarase, and phosphoenolpyruvate carboxylase activities (mg^{-1} chlorophyll) as

did whole-leaf extracts, indicating they were relatively free of peroxisomal, mitochondrial, or cytoplasmic components.

ACCase activity as measured by acetyl-CoA-dependent $H^{14}CO_3^-$ (ICN, 2.07 GBq $mmol^{-1}$) incorporation into acid-stable product previously shown to be malonyl-CoA (J.D. Burton et al., Pesticide Biochemistry and Physiology, 34, 76-85 (1989)). Assays of desalted purification fractions or crude, desalted tissue extracts contained up to 50 and 25% (v/v) enzyme, respectively. In some experiments methylcrotonyl-CoA or propionyl-CoA were substituted for acetyl-CoA (E.S. Wurtele et al., Archives of Biochemistry and Biophysics, 278, 179-186 (1990)). Avidin (10 U mL^{-1}) was included in some assays. Herbicide inhibition assays contained 1% (v/v) ethanol plus or minus 1 μM haloxyfop (2-[4-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]oxy]phenoxy] propanoic acid, Dow Chemical Co. analytical grade racemic mixture) or 10 μM sethoxydim (2-[1[(ethoxylmino)butyl]-5-[2-(ethylthio)-propyl]-3-hydroxy-2-cyclohexene -1 -one, Li salt, BASF Corp. technical grade). Data are means plus standard error of three assays.

Protein concentrations were determined in duplicate with the Bio-Rad Coomassie blue dye-binding assay as described by the manufacturer, using BSA as the standard.

Centrifuged crude extracts and proteins in purification fractions and immunoprecipitation supernatants were separated by SDS-PAGE in 6 or 7.5% gels as previously described (W.B. Parker et al., Plant Physiol., 92, 1220-1225 (1990)). Purification fractions were precipitated in 10% (v/v) TCA, washed with 80% (v/v) acetone, and air-dried 10 minutes prior to electrophoresis. Proteins in gels were stained with silver (J. Heukeshoven et al., Electrophoresis, 6, 103-112 (1985)). High molecular weight protein standards for SDS-PAGE (Pharmacia) were used to estimate polypeptide masses.

The four-step purification procedure shown in Table IV typically yielded 30 to 190 μg of highly purified ACCase from 50 grams (fresh weight) of maize inbred A619 or B73 seedling leaves. ACCase activity in the crude supernatant fraction precipitated between 30 and 40% saturation with $(NH_4)_2SO_4$, which appeared to increase total ACCase activity approximately 38%. Crude extract components might have depressed the reaction rate shown in Table IV

because the assay mixture contained 50% enzyme (v/v). In tests of fractions from another purification, enzyme velocity was proportional to enzyme concentration in assay mixtures containing up to 25% (v/v) crude extract, but 50% (v/v) mixtures were not tested. ACCase activity eluted from the Sephacryl S-300 gel filtration column slightly after the green void peak. Approximately 56% of the S-300 fraction ACCase activity was recovered from the Blue Sepharose column, primarily in the initial ATP-containing fractions (12.5 mL). Both 10 mM $NaHCO_3$ and 4.25 mM $MgCl_2$ (1- and 0.85-fold standard assay concentrations, respectively) were included in the Blue Sepharose buffer because they improved the total and specific ACCase activity remaining after batch absorption to Blue Sepharose beads, elution with ATP, and desalting into extraction buffer minus PMSF. Neither $NaHCO_3$ nor $MgCl_2$ improved enzyme stability of crude extracts. Mono-Q anion-exchange chromatography resulted in separation of two ACCase activity peaks which eluted at approximately 210 mM (designated ACCase II) and 250 mM KCl (designated ACCase I), as previously observed for a hybrid maize variety (J.L. Howard et al., FEBS Lett., 261, 261-264 (1990)). ACCase I comprised about 85% of the total activity recovered from the column (29% of the original crude extract activity) and had high specific activity (Table IV). The specific activity of ACCase II was less than 30% that of ACCase I. Both activities were inhibited >90% by avidin, as previously reported (J.L. Howard et al., FEBS Lett., 261, 261-264 (1990)). The mass of native ACCase I was estimated to be approximately 490 kD by gel filtration on Superose 6.

TABLE IV

Purification of ACCase I From
Maize Inbred A619 Seedling Leaves[a]

All fractions were desalted into S-300 buffer and assayed for protein and acetyl CoA dependent incorporation of $[^{14}C]HCO_3^-$ into acid-stable products.

Step	Protein	Activity	Specific Activity	Fold Purification	Activity Yield
	mg	*units*[b]	*units/mg*		%
Crude extract	215	2.45	0.0114	1	100
30-40% $(NH_4)_2SO_4$	45.1	3.37	0.0748	6.56	138
S-300	10.7	3.35	0.313	27.5	137
Blue Sepharose	1.50	1.86	1.24	109	76
Mono-Q (ACCase I)	0.130	0.720	5.54	486	29

[a] Data are from one purification experiment starting with 50 g fresh weight of tissue and are representative of data obtained for eight purifications.

[b] Unit = 1 μmol acid-stable product min^{-1}.

B. Formation and Specificity of Antibodies to ACCase

Antibodies are sensitive reagents that allow for the identification of gene products from cDNA and other cloned genes. Antibodies to purified ACCase were prepared and used to screen for cDNA clones encoding all or a portion of a gene for ACCase.

Antiserum to maize ACCase was obtained by immunizing a female New Zealand White rabbit (Egli et al., Plant Physiol., 101, 499 (1993)). An intramuscular injection of 100 μg of Mono-Q-purified, SDS-denatured ACCase I in Freund's complete adjuvant was followed by subcutaneous injections of 20 to 100 μg of gel-purified ACCase I polypeptide in acrylamide plus incomplete adjuvant every 4 to 6 weeks, for a total of six injections. Serum was stored at -20°C in 0.02% (w/v) NaN_3.

For Western blots, proteins in SDS gels were electrophoretically transferred to Immobilon (W.B. Parker et al., Plant Physiol., 92, 1220-1225

(1990)) for 1 hour at 20 V in a Bio-Rad Transphor semi-dry blotter and then stained with Ponceau S (E. Harlow et al., "Antibodies - A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1988)). Destained blots were blocked with Tris-buffered saline plus 0.5% (v/v) Tween-20 (Bio-Rad), and 10% (w/v) bovine serum (for antiserum blots only). ACCase and biotinylated proteins were detected with immune serum (1/10,000) plus goat anti-rabbit IgG-alkaline phosphatase conjugate or with avidin-alkaline phosphatase (W.B. Parker et al., Plant Physiol., 92, 1220-1225 (1990)). Blots were repeated at least three times.

For immunoprecipitations, equal ACCase activities (0.58 nmol min^{-1}) in crude extracts were desalted into S-300 buffer containing 0.1 M KCl and incubated 1 hour at 25°C with 16 µL buffer or with 16 µL serum consisting of 0 to 100% ACCase antiserum in preimmune serum. Immune complexes were incubated 1 hour at 25°C with a 2-fold (IgG binding) excess of Protein A-agarose and then microfuged 5 minutes to obtain immunoprecipitation supernatant fractions. ACCase activity of supernatants was expressed as a percent of the 100% preimmune serum control. Data are means plus SE of three replicate assays for each of two sets of extracts.

Western blots and silver-stained gels of purification fractions separated by 7.5% SDS-PAGE showed that neither ACCase I nor ACCase II Mono-Q fractions contained biotinylated polypeptides smaller than 212 kD. A polypeptide > 212 kD was the primary protein component of the ACCase I Mono-Q fragment (Fig. 5). The ACCase II fraction contained a biotinylated polypeptide > 212 kD and a large amount of a 55 kD non-biotinylated polypeptide. Fractions from earlier purification steps contained additional biotinylated proteins of approximately 74, 75, and 125 kD (Fig. 5).

To better compare the biotinylated polypeptides > 212 kD in ACCase fractions I and II, we used 6% SDS-PAGE, which showed that the mass of ACCase II was approximately 8 kD less than that of ACCase I. Molecular masses were estimated to be 219 kD (ACCase II) and 227 kD (ACCase I), based on comparisons with polypeptide standards and the observation (N.R. Palosaari, Plant Physiol., 99(S), 359 (1992)) that, on Phastgels (Pharmacia), ACCase I polypeptide was slightly smaller than dodecameric horse spleen ferritin (238 kD;

M. Heusterspreute et al., FEBS Lett., 129, 322-327 (1981)). All purification fractions through the Blue Sepharose step contained both ACCase I and II polypeptides. Rapid extraction of leaves in buffer containing five additional protease inhibitors, or a 4 hour incubation of extracts at 25°C, had little or no effect on the relative amounts of the two polypeptides, suggesting that ACCase II is not a breakdown product of ACCase I.

Antiserum to ACCase I strongly recognized the ACCase I polypeptide in crude extracts and showed little or no recognition of ACCase II polypeptides. No bands were recognized by preimmune serum. Assuming that avidin binds similarly to ACCase I and II polypeptides, it appears that the amount of ACCase II on the Western blot was slightly less than the amount of ACCase I. However, the relative staining with antibody compared to avidin indicated that the antibody had significantly less affinity for ACCase II than ACCase I.

To determine whether the same ACCase polypeptides were expressed in different maize cell types, proteins in mesophyll chloroplasts and crude extracts of leaves, endosperm tissue, embryos, and BMS cells were separated by SDS-PAGE. All preparations contained a predominant biotinylated polypeptide of approximately 227 kD (ACCase I) that was strongly recognized by ACCase antiserum or avidin. Similar 227 kD band densities were observed when gel lanes were probed with either avidin or ACCase antiserum. The 219 kD ACCase II polypeptide was readily detected in leaves only by avidin binding, but was in low abundance or not detected in extracts from other tissues. Only the 227 kD ACCase I polypeptide was detected in purified mesophyll chloroplasts, however, suggesting that the 219 kD ACCase II polypeptide is localized elsewhere in mesophyll cells or in other cell types of young leaves. ACCase activity and a > 212 kD biotinylated polypeptide(s) were also found in bundle sheath strand extracts, but low yields prevented us from determining the type of ACCase present. Two other major biotinylated polypeptides of 75 and 74 kD were found in all tissues. Other non-biotinylated proteins of 66 kD (faint) and 55 kD were also recognized by ACCase antiserum. The 55 kD polypeptide was only found in leaves; it was also present in both ACCase I and II Mono Q

fractions (Fig. 5) and was identified as the Rubisco large subunit based on its comigration with protein immunoprecipitated by spinach Rubisco antiserum.

ACCase antiserum immunoprecipitated at least 75% of ACCase activity from crude, desalted extracts of leaves, endosperm tissue, embryos, and BMS cells (Fig. 6), indicating that most of the ACCase activity in these tissues is immunologically related to the ACCase I polypeptide of leaves. Less activity was precipitated from leaves (75%) than from other tissues, particularly embryos (98%). Compared to immunoprecipitation, inhibition of ACCase activity by antiserum in solution was less than 20% as effective in reducing ACCase activity.

The substrate specificity of ACCase from different purification fractions was examined to compare [^{14}C]HCO_3^- incorporation in the presence of different acyl-CoA substrates. Both ACCase I and II utilized propionyl-Co-A 40 to 50% as rapidly as acetyl-CoA at 50 to 500 μM substrate even though they contained no biotinylated polypeptides (Figure 5) the size of known propionyl CoA carboxylases (70 to 75 kD; see E.S. Wurtele et al., Archives of Biochemistry and Biophysics, 278, 179-186 (1990)). Activities in the presence of both acetyl-CoA and propionyl-CoA (250 or 500 μM each) were approximately 90 (ACCase I) to 130% (ACCase II) that of 500 μM acetyl-CoA alone. Crude leaf extracts utilized propionyl-CoA and methylcrotonyl-CoA 60% as efficiently as acetyl-CoA. Methylcrotonyl CoA carboxylase activity was reduced 85% by gel filtration and was completely removed by Blue Sepharose affinity chromatography.

ACCase I and II differed significantly in their inhibition by either haloxyfop or sethoxydim (Fig. 7). Acetyl-CoA or propionyl-CoA-dependent $H^{14}CO_3^-$ incorporation by ACCase I was strongly inhibited (65 to 80%) by 1 μM haloxyfop or 10 μM sethoxydim, while ACCase II activity was inhibited less than 50% for all herbicide/substrate combinations examined.

C. Cloning and Identification of Maize cDNA Clones Encoding ACCase

Maize cDNA clones encoding a portion of the ACCase game were identified by screening a DNA library generated from maize. The cDNA clones were used to identify the sequence of the ACCase gene and to identify the genomic DNA fragments encoding the gene or genes for ACCase.

A λ gt11 cDNA library from maize inbred A188 seedlings was prepared by standard method for oligo-dT priming, as described for pea cDNA. (Gantt and Key, Eur. J. Biochem., 166:119-125 (1987). Plaque lifts of the maize cDNA library were screened with maize ACCase antiserum (Egli et al., Plant Physiol., 101, 499 (1993)) to identify plaques expressing ACCase-like proteins, as described by Sambrook et al., cited supra. (1989). The initial screen of 800,000 plaques yielded 120 positives. Rescreening and plaque purification reduced the number of positives to 14. All 14 clones bound ACCase antibodies that, when eluted from plaque lifts (J. Hammarback et al., J. Biol. Chem., 265:12763 (1990)), recognized a 227-kD biotinylated polypeptide on SDS-PAGE western blots of embryo and leaf crude extracts. The strongest western blot reaction was obtained with cDNA clone #15-14. The six best clones were digested with EcoRI to excise maize cDNA inserts. Total insert sizes ranged from 1.2 to 5.1 kb indicating the clones most likely did not contain the full coding sequences for the mature 219-kD and 227-kD ACCase polypeptides (minimum estimates of 6.1 and 6.3 kb, respectively).

Clone #15-14 contained three EcoRI fragments of 2.0, 1.2 and 0.23 kb shown in Figure 8. Southern blots showed that the 1.2 and 2.0-kb fragments of clone #15-14 each hybridized to different fragments in the other five clones, with the exception of clone #4-4 which only contained a 1.2-kb fragment. The six maize cDNA clones contained EcoRI fragments that hybridized to a large transcript (ca. 7.8 kb) on Northern blots of total RNA from maize leaves, embryos and endosperm (Fig.9). BMS cell culture RNA also contained a 7.8 kb transcript, but the hybridization signal is not evident on this exposure (Fig. 9). The relative abundance of the 7.8-kb transcript in embryos was higher than the other sources which is consistent with their ACCase activity.

The three EcoRI fragments were subcloned from cDNA clone #15-14 into BlueScript vector and sequenced by the dideoxy chain termination method (Sequenase 2.0 USB) initially using T3 and T7 primers and then oligonucleotide primers based on insert sequence. A clone #16-6 was also sequenced in a similar manner. Clone #16-6 included three EcoRI fragments of 3.1 kb, 1.2 kb, and 0.23 kb and had additional sequence located upstream from that of clone #15-14. After comparing the sequence and determining that the

sequence was the same, the additional 1.2 kb sequence at the 5′ end was sequenced.

Clone #18-5 was sequenced in a similar manner. Clone #18-5 included 3.9 kb, 1.2 kb, and 0.23 kb EcoRI fragments and contains an additional 1.9 kb 5′ sequence upstream from clone #15-14. Subclone #18-5I (3.9 kb EcoRI fragment) has been deposited with the American Type Culture Collection and given Accession No. 69236.

GenBank, PIR-29, and Swiss-Prot 19 data banks have been searched for amino acid homology with the corresponding amino acid sequences of the three subclones of clone #18-5. Peptide sequences corresponding to the maize cDNA subclones had higher similarity to chicken, rat, and yeast ACCases than to any other peptide sequence in the data banks. Figure 8 illustrates the relative organization of the 3.9, 1.2 and 0.23-kb EcoRI fragments of clone #18-5 and their co-linearity and extent of amino acid identity with chicken ACCase cDNA sequence. This comparison shows that the maize clone #18-5 has a large region near the 3′ end with high amino acid identity (40 to 61%) to chicken ACCase, a longer region with 23% identity in the middle of the 3.9-kb sequence, and a short region with 52% identify near the 5′ of the 3.9 kb sequence.

Portions of the sequence of the #18-5I subclone have been identified as encoding domains of the ACCase enzyme of functional significance. Those functional regions include a fragment that spans the presumed transcarboxylase active site in the enzyme having the following presumed sequence SEQ ID NO: 2:

1112-856

GTT CCT GCA AAC ATT GGT GGA CCT CTT CCT ATT ACC AAA CCT CTG GAC
CCT CCA GAC AGA CCT GTT GCT TAC ATC CCT GAG AAC ACA TGC GAT CCA
CGT GCA GCT ATC TGT GGT GTA GAT GAC AGC CAA GGG AAA TGG TTG GGT
GGT ATG TTT GAC AAA GAC AGC TTT GTG GAG ACA TTT GAA GGA TGG GCA
AAA ACA GTG GTT ACT GGC AGA GCA AAG CTT GGA GGA ATT CCT GTG GGC
GTC ATA GCT GTG GAG ACA

This functional domain is contained in the sequence 1112 to 856 base pair from the 3′ stop codon or carboxy terminus region of the ACCase coding sequence of maize. This transcarboxylase active sequence is also present in clone #15-14.

Another functional region that has been identified spans the 12 base pair sequence encoding the biotin binding site having the following peptide sequence SEQ ID NO: 3:

5′ GTT ATG AAG ATG 3′
Val Met Lys Met

The biotin binding site is encoded approximately 30% in from the 5′ (N-terminus) end of rat, chicken and yeast ACCase genes. These functional domains are useful in mapping and further identifying other cDNA and/or genomic fragments encoding ACCase genes.

The cDNA clones encoding portions of the acetyl CoA carboxylase genes are useful to identify the sequence of the gene or genes and are useful as probes to locate the genomic copies of the gene or genes. Because the ACCase antibodies used to screen the λ gt11 library recognize both the 219 and 227 kD ACCase polypeptides, it has not been determined which polypeptide is encoded by these less than full length clones. It is likely that the majority of the clones encode the 227 kD polypeptide since that polypeptide is more abundant in the leaf tissue source of the DNA library and the antibodies have a higher affinity for the 227 kD ACCase polypeptide.

EXAMPLE VI

Isolation and Sequencing of Genomic Encoded ACCase Genes and a Complete cDNA Sequence of a Maize ACCase Gene

The maize genome has been analyzed to identify copy number and location of the genomic copies of ACCase gene or genes. Four distinct types of maize ACCase genomic clones have been identified, termed A1, A2, B1 and B2 (see below).

To obtain genomic copies of ACCase genes, a maize B73 genomic library (Clontech, Palo Alto, CA) was screened with the 2 kb subclone from #15-14 and several clones of about 15 kb were identified as having homology to the ACCase cDNA. Restriction mapping and partial sequence analysis revealed two types of genomic clones (Type A and Type B) that differed

in restriction sites and in their position relative to the ACCase partial cDNA sequence as shown in Figure 8.

The 2.5 kb EcoRI-SalI fragment (#16) from the Type A genomic clone and the 3.0 kb EcoRI-EcoRI fragment (#34) from the Type B genomic clone were shown to hybridize to the 3.9 kb probe from #18-5 and were subcloned into the Bluescript vector and sequenced. Approximately 1.5 kb of DNA sequence from the genomic Type A 2.5 kb fragment were 100% identical to coding sequence from the 3.9 kb cDNA subclone #18-5I described in Example V; the remaining sequence exhibited no identity with the cDNA clone and presumably represents a noncoding intron sequence. A 350 nucleotide sequence derived from the genomic Type B 3.0 kb fragment was about 95% identical to the cDNA clone indicating that its coding sequence differs from that of genomic Type A. These results also indicate that the maize genome encodes at least two different genes encoding a polypeptide having acetyl CoA carboxylase activity.

To identify and clone the remainder of the gene representing the amino-terminus of maize ACCase, additional regions from the Type A genomic clone have been subcloned and partly sequenced. The 3.5 kb HindIII-HindIII fragment (#28) has been sequenced for about 400 nucleotides from each end. The 3′ end of #28 shows significant homology to the amino acid sequence from the chicken sequence located about 0.5 kb from the start of the chicken gene.

The complete sequence for fragment #28 can be obtained and analyzed to determine whether it contains the 5′ end of the ACCase coding region. The start of the transcribed region, and thus the likely start of the coding region for ACCase, can be identified by using the genomic clones in RNAse protection analysis (J. Sambrook et al., "Molecular Cloning - A Laboratory Manual," Cold Spring Harbor Press, Cold Spring Harbor, New York (1989)). Based on sequence data from the genomic clone, alignment, as shown in Figure 8, with sequences of other ACCases and identification of potential open reading frames, oligonucleotide primers can be constructed to synthesize cDNA molecules representing the amino terminus of the ACCase gene. These molecules can be hybridized to genomic Type A DNA fragments such as #28 and the nonhybridizing portions digested with S1 nuclease. The end of the protected fragment are determined by analysis on a DNA sequencing gel.

To synthesize the remaining coding region between the end of the cDNA clone #18-5 and the start of transcription, two oligonucleotide primers were synthesized. Primer 1 is complementary to the DNA sequence: (SEQ ID NO:4)

5′ GCCAGATTCC ACCAAAGCAT ATATCC 3′

near the 5′ end of cDNA subclone #18-5I and can be used as a primer for synthesis of cDNA molecules from maize seedling, leaf or embryo RNA.

A primer corresponding to a DNA sequence near the transcription start site can be used in combination with primer 1 for the amplification of DNA by the polymerase chain reaction (PCR). Several independent clones are then sequenced and their sequences compared to the known sequence of the Type A genomic clone to determine the exact coding sequence corresponding to that maize gene for ACCase. A similar strategy can be used to obtain the complete coding sequence for genomic Type B ACCase.

The remaining cDNA sequence was obtained by three successive rounds of RT-PCR using oligonucleotide primers based on genomic apparent exon (5′) and known cDNA (3′) sequences. The primers used to amplify nucleotides 1-240 of the cDNA were 28sst-a5+ (SEQ ID NO. 7) and 28sst-6at3+ (SEQ ID NO:8), nucleotides 217-610 of the cDNA were 28sst-5+ (SEQ ID NO:9) and 28-2t3+ (SEQ ID NO:10), and nucleotides 537-2094 of the cDNA were ACCPCR5′ (SEQ ID NO:11) and I55′ (SEQ ID NO:30) (Table V). PCR products corresponding to nucleotides 1-240, 217-610, and 537-2094 of the final sequence were cloned into PCR-script (Stratagene).

TABLE V

cDNA Position	5′ primer designation	5′ primer sequence	3′ primer designation	3′ primer sequence
nt-1-240	28sst-a5+	GGTCTTCAATTGTGCTGTCTGG (SEQ ID NO:7)	28sst-6at3+	CCTTGACGAACAGACTGGCTGTGC (SEQ ID NO:8)
nt 217-610	28sst-5+	CACAGCCAGTCTGTTCGTCAAGG (SEQ ID NO:9)	28-2t3+	CCTCTACGTAATTGGTCAGC (SEQ ID NO:10)
nt 537-2094	ACCPCR5′	CATAGCTATGGCAACTCCGG (SEQ ID NO:11)	I55-	GGATATATGCTTTGGTGGAATCTGGC (SEQ ID NO:30)

The original 5.4-kb cDNA clone #18-5 and PCR products from at least three individual PCR per oligonucleotide pair were sequenced in both directions by the dideoxy chain-termination method, using either Sequenase II (U.S. Biochemicals) or ABI 373 (Applied Biosystems, Inc.) protocols. No sequence differences were found in regions of clone overlaps. The complete sequence of the cDNA of maize ACCase (nucleotides 1-7470 SEQ ID NO:5) and its corresponding amino acid sequence (amino acids 1-2325 SEQ ID NO:6) are shown in Figures 13 and 14. The 7470 bp cDNA includes a 459 nucleotide 3′ untranslated region and 36 nucleotides of 5′ untranslated sequences.

The first Met codon in the cDNA (nucleotides 37-39) was identified as the start codon based on its similarity to consensus initiation sequences (Kozak, J. Cell. Biol., 108, 229 (1989); Lutcke et al., Embo. J., 6, 43 (1987)). An in-frame stop was found in the genomic sequence 6 nucleotides upstream of the sequenced cDNA, and RT-PCR analysis of this region suggested that the in frame stop codon was also present in the cDNA. The 3′ end of the coding sequence was defined by two stop codons present in the large open reading frame after nucleotide 7011. The translated coding sequence predicted a polypeptide of 2325 amino acids (257 kD; SEQ ID NO:6) which was 79 to 81% identical to the multifunctional (MF) ACCases from alfalfa (Shorrosh et al., Proc. Nat'l. Acad. Sci., 91, 4323 (1994)) and wheat (Gornicki et al., Proc. Nat'l Acad. Sci., 91, 6860 (1994)), and to a 118-amino acid predicted polypeptide of a rice expressed sequence tag (Genbank accession # D39099, T. Sasaki), but only 53 to 55% identical to ACCase from other eukaryotes.

In a pileup alignment of plant ACCases (Genetics Computer Group, Madison, Wisconsin), Met 1 of both maize and *Brassica napus* ACCases was located about 130 amino acids upstream of the conserved sequence VDEFCKALGG, compared to only 25 amino acids upstream for other plant ACCases. The predicted 2325 amino acids of maize ACCase contains a biotinylation site at position 806, within the conserved MKM motif (Ton et al., Eur. J. Biochem., 215, 687 (1993)). The arrangement and amino acid sequence of binding sites (Shorrosh et al., Proc. Nat'l. Acad. Sci., 91, 4323 (1994)) for ATP (amino acids 318-333), biotin (amino acids 799-811; biotin at 806),

acetyl-CoA (amino acids 1952-1961), and carboxybiotin (amino acids 1662-1711) were highly conserved among all MF ACCases.

EXAMPLE VII

Characterization of other Genomic Clones

The initial restriction fragment length polymorphism (RFLP) analysis of EcoRI-digested total DNA from three maize inbred lines showed one band when probed with the 2 kb subclone from #15-14 (internal to gene) and two bands when probed with the 1.2 kb subclone (near the 3′ end of the gene). Fragments homologous to the 2 kb probe were monomorphic and the more intense of the two bands hybridizing with the 1.2 kb probe was dimorphic. As discussed in Example V, these results support the view that maize contains at least two distinguishable ACCase genes and that they may be quite similar for much of the coding region. Additional genomic Southern blots of a set of recombinant inbred lines were used to map polymorphisms for the ACCase probes to maize chromosomes. One polymorphism was mapped to the short arm of chromosome 2; other polymorphisms were not evident in these initial tests to identify a chromosomal location for other maize ACCase genes.

The isolation and restriction mapping of additional genomic clones from a B73 genomic library (Clontech) resulted in the identification of four different types of clones termed A1, A2, B1 and B2 (Figures 16-19) which had 96% nucleotide sequence identity. Types A and B correspond to previously published pA3 and pA4 cDNAs (Ashton et al., Plant Mol. Biol., 24, 35 (1994)) and differ from pA3 and pA4 by ~ 4% in their coding sequences.

Type A and B genomic clones have linear sequence homology except for an insertion in an intron of the Type B genes about 1400 bp 3′ of the A1(SEQ ID NO:5) translation start site. Analysis of the insert boundaries revealed a 3-bp target site duplication and a 6-bp direct repeat, and further sequence analysis showed the presence of two new and unique LINE elements (Long Interspersed Nuclear Elements) in B1 and B2. Mammalian LINE elements are highly abundant (10^4 to 10^5 copies), 6 to 7 kb long, and have frequent 5′-end deletions and an A-rich 3′ terminus. They are flanked by short direct repeats, and contain two ORFs, one encoding a reverse transcriptase. Three LINE elements (Cin4, 50-100 copies in maize; del2, 250,000 copies in

lily; BNR1, 2-5% of genome in sugarbeet) have been described in plants (Leeton et al., Mol. Gen. Geneti., 237, 97 (1993); Schmidt et al., Chromo. Res., 3, 335 (1995); Schwarz-Sommer et al., EMBO J., 6, 3873 (1987)). Maize ACCase B1 has one unique LINE element and B2 has two. The two B2 LINE elements were characterized by differences in their reverse transcriptase sequence. The B genomic clone inserts have characteristic LINE features including cysteine motifs and a possible polyA tail, and high abundance. The LINE insert also has been found in an intron of the maize *Shrunken-2* gene (Hannah et al., Plant Physiol., 98, 1214 (1992)).

The partial nucleotide sequence (3489 nucleotide) of a Type A1 ACCase genomic clone is shown in Figure 16 (SEQ ID NO:12). The clone is a HindIII fragment which includes nucleotides 1-931 of the cDNA in Figure 13 (SEQ ID NO:5), and the first four introns within the coding region, at positions 240 (460 nucleotides), 296 (480 nucleotides), and 872 (76 nucleotides) of SEQ ID NO:5. The clone also has 1395 nucleotides 5′ to the cDNA of SEQ ID NO:5 (i.e., 1431 nucleotides 5′ of the translational start at nucleotide position 1432).

The partial nucleotide sequence (1328 nucleotide) of another Type A clone is shown in Figure 17 (SEQ ID NO:13). The partial sequence is all 5′ untranslated sequence and contains a 7 base insert between nucleotides 279-290, but is otherwise identical to SEQ ID NO:12.

The partial nucleotide sequence of six Type A2 clones is shown in Figure 18 (1565, 1168, 638, 558, 976 and 852 nucleotides; SEQ ID NOs 14, 15, 16, 17, 18 and 19, respectively).

Within the A1-A2 clone pair, identified differences are in introns and 5′ UTR sequences. The A2 genomic clone is weakly amplified with Type A1 PCR primers specific for the 5′ UTR if the 3′ primer employed is for a conserved amino acid sequence found in all ACCases (e.g., 28sst-110F, ACTGTGCGTTTGAGAAGGTC, SEQ ID NO:28, and 28sst-2T3+, CCTCTACGTAATTGGTCAGC, SEQ ID NO:29). The A2 amplified product is the same size as that from the Type A1 genomic clone, and restriction analysis indicated a difference in sequence from A1. Sequence differences in the 5′ region should provide a means to distinguish between expression of A1 and A2 ACCase genes and to determine whether A2 also encodes a CTP.

The partial nucleotide sequence (231, 207 and 180 nucleotides) of three Type B clones is shown in Figure 19 (SEQ ID NOs 20, 21 and 22, respectively).

The cDNAs corresponding to genomic clones A2, B1 and B2 are cloned and sequenced in a manner similar to that described above. The derived amino acid sequences are aligned with known ACCase sequences. If putative CTP sequences are identified, functionality is tested as described below. Also if the tissue specificity and developmental timing of expression differ for different ACCase genes, the sequences of the promoter regions of the corresponding genomic clones are compared. Gene-specific probes for specific ACCase genes can provide more information on their roles in lipid synthesis (plastid and cytoplasmic isoforms), secondary metabolism (cytoplasmic isoforms), and herbicide resistance (likely plastid isoforms).

A 3′ Type A1 ACCase cDNA probe mapped to chromosome 2S (Egli et al., Maize Genetics Newsletter, 68, 92 (1994)) and to 10L (Caffrey et al., Maize Gen. Coop., 69, 3 (1995)). Two 5′ Type A1 cDNA probes which span the transit peptide mapped to chromosome 2S in the same location as the 3′ probe (see maize genetic map, 1996 version, Maize Genomic Database). PCR primers 28sst-97F (CCTTTTTATGGCACTGTGCG, SEQ ID NO:25) and 28sst-6t3+; (CATCGTAGCCTATATGAGGACG, SEQ ID NO:26) located in non-coding regions of A1 that span the chloroplast transit peptide were used to amplify a B73 chromosome-specific product which segregated with the resistance trait. A nearby 5′ primer (28sst-a5+, SEQ ID NO:7) amplified all genotypes and functioned as a positive control. Herbicide resistance due to the *Acc1-S3* mutation segregates (29/29 individuals to date) with production of a Type A 5′ end-specific PCR product derived from the mutant parent while herbicide sensitive plants lack the transit sequence (15/17 progeny). Two individual plants which contained B73-specific DNA at this location died of unknown causes while grown in the presence of herbicide.

Mutations in maize that confer resistance to cyclohexanedione and aryloxyphenoxypropionate herbicides by means of an altered ACCase target are found at two non-allelic loci, *Acc1* and *Acc2*. A1 and A2 appear to encode plastidic ACCases and correspond to the *Acc1* and *Acc2* herbicide resistance

loci. *Acc2* has been mapped to 10 L (VanDee, M.S. Thesis, University of Minnesota (1994)). *Acc1* is the site of five allelic mutations including *Acc1-S2* and *-S3* (Marshall et al., Theor. Appl. Genet., 83, 435 (1992)), and has been mapped to chromosome 2.

Only one plastidic ACCase polypeptide was identified by SDS-PAGE of maize leaf extracts, although 2-D gel analyses might provide evidence for a second, highly similar isoform. Of the two ACCase isoforms, only ACCase I shows altered herbicide inhibition in *Acc1-S2* mutants, and most of the ACCase activity in leaves and developing embryos is herbicide-resistant and thus attributed to the *Acc1-S2* gene product.

Although a 3′ ACCase probe has been mapped both to 10L near *Acc2-S5* and to 2S, the conserved sequence of ACCase genes and lack of polymorphism in multiple bands complicates identification of genes encoded at these loci. The Type A1 ACCase gene is probably located on chromosome 2, since (i) 5′ untranslated and chloroplast transit peptide probes from Type A1 hybridize to two bands (dark and light) in maize inbreds, and (ii) analysis of maize-oat addition lines carrying maize chromosomes 2 through 9 indicates the dark band is on chromosome 2 and the light band is on chromosome 1 or 10.

Type B ACCase genes are likely to encode cytosolic isoforms. Given that cytosolic malonyl-CoA is a precursor in the synthesis of many secondary metabolites including flavonoids (e.g. maysin, a corn silk component associated with corn earworm resistance), these cytosolic ACCases can have agronomic utility.

Northern blot analysis of total maize RNA with an ACCase probe (nucleotides 3400-5932) showed a single 8.3 kilobase band. To determine whether the expression of ACCase RNAs was developmentally regulated, blots of total RNA from 16 to 42 DAP (days after pollination) embryos were probed with an ACCase cDNA fragment. Transcript abundance peaked about 23 DAP and the steady state pattern was similar to *in vitro* ACCase enzyme activities and protein measured from developing embryos. Type A- and B- specific ^{32}P-CTP-labeled antisense transcripts were 780 nt long (662 nt of ACCase sequence + 118 nt of vector/promoter sequence) and were identical except for 15 base mismatches scattered along their length. Each antisense transcript was

hybridized to total RNA from embryos at 16, 20, 23, and 42 DAP and digested with RNAse A/TI mixture to yield a 662-base fragment specific to the probe used. The results showed that the Type A transcript was more abundant than Type B at all tested stages, and that only Type A remained high in older embryos. Types A and B had similar expression patterns and peaked around 20-23 DAP. The ratio of Type A:B mRNA in leaves was about 2:1, similar to its relative abundance in cDNA expression libraries.

EXAMPLE VIII

Expression of the Maize ACCase Chloroplast Transit Peptide

The N-terminus of the predicted maize ACCase polypeptide is longer than that of predicted cytosolic ACCase isoforms and has several characteristics typical of chloroplast transit peptides within the first approximately 73 amino acids of the predicted N-terminal sequence. The CTP cleavage site motif is not found in the putative maize ACCase CTP, although only about 30% of known CTPs contain this consensus sequence (Gavel and von Hejne, FEBS Lett., 261, 455 (1990)). However, the maize ACCase N-terminus appears to have several other properties typical of known CTPs: (1) a lack of acidic residues in amino acids 1-49, (2) high Ser + Thr content (69% within amino acid residues 23-35), (3) an R-rich region between S- and D-rich regions in amino acid residues 36-49, and (4) a predicted turn →β sheet within amino acid residues 58-73 (von Hejne and Nishikawa, FEBS Lett., 278, 1 (1991)).

The ability of the amino acid sequence contained within the N-terminal 100 amino acids of the translated maize acetyl-CoA carboxylase (ACCase) cDNA to direct the N-terminal portion of the maize ACCase biotin carboxylase domain into chloroplasts was tested *in vitro* by methods used extensively in the literature (see Cline et al., J. Biol. Chem., 260, 3691 (1985); Lubben and Keegstra, Proc. Nat'l. Acad. Sci., 83, 5502 (1986)). The criteria for import was that (1) *in vitro*-synthesized, ^{35}S-labeled protein was imported into chloroplasts, and (2) the transported protein was smaller than the original translation product, by an amount which corresponds to the removal of the expected CTP. Import studies utilized either maize or pea chloroplasts. Pea chloroplasts have been reported to correctly import proteins from many different species, including maize (Nieto-Sotelo et al., Plant Physiol., 93, 1321 (1990)).

Alternatively, the function of the putative maize ACCase CTP is tested by inserting the first 258 coding nucleotides of maize ACCase in frame with and 5′ of a GUS reporter gene in pBI221 (Clontech). This construct and the pBAR plasmid are used to co-transform maize "Black Mexican Sweet" suspension cells by particle bombardment. Basta-resistant transformants are selected, and GUS activity and/or protein is assayed in surviving cultures or in plasmids isolated from transformants.

A partial ACCase construct consisting of nucleotides 1-833 of SEQ ID NO: 5 including the putative CTP (nucleotides 37 to 256) and the first domain within the biotin carboxylase region (identified by amino acid sequence comparison with E. coli biotin carboxylase; see Waldrop et al., Biochem., 33, 6249 (1994)) was amplified by PCR and cloned into the EcoRV site of PCR-script (Stratagene) to create the plasmid pBCN1. A corresponding plasmid lacking CTP sequences (nucleotides 278-833) was also made (-pBCN1). The protein encoded by -pBCN1 begins at amino acid residue 83 (Val → Met).

Constructs were transformed into E. coli SURE cells (Stratagene). Restriction analysis of pBCN1 with BamH1 and HindIII indicated that the 5′ end of the ACCase was located adjacent to the T7 RNA polymerase binding site in PCR-Script. A partial sequence of pBCN1 obtained by using the T7 sequencing primer and the ABI373 automated sequencing protocol confirmed this orientation and showed that the pBCN1 insert sequence was identical to maize ACCase cDNA for at least the first 300 nucleotides and that it included the maize ACCase Met 1 ATG. An acyl carrier protein clone containing a CTP (spinach ACPII, a gift of Dr. John Ohlrogge, Michigan State University) can be used as a positive control. These constructs are used for *in vitro* transcription, translation, chloroplast import, and SDS-PAGE analysis of products in the same manner as pBCN1.

Purified pBCN1 was digested with EcoRI to linearize the plasmid at the 3′ end of the BCN1 insert, electrophoresed in 1.5% agarose, and the plasmid band at approximately 3.8 kb was excised and Gene-Cleaned (BioLab 101). The purified band was digested with 20 μg proteinase K to remove any residual RNAse, extracted with phenol and then chloroform under RNAse-free conditions. DNA content was estimated by ethidium bromide fluorescence in

droplets, relative to λ DNA standards (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed. (1989). One μg of pBCN1 DNA was transcribed into capped RNA with the T7/mMessage mMachine kit (Ambion). Uncapped transcripts (Sp6 RNA polymerase; Promega) of pea RUBISCO small subunit (SSU; Anderson et al., Biochem. J., 240, 709 (1986)) were also transcribed. RNA yield was estimated by determining the % incorporation of a ^{32}P-ATP (Amersham) into a precipitable product, according to the Ambion kit instructions. Electrophoresis and autoradiography of ^{32}P-labeled product showed that it contained a single RNA band of approximately 895 nucleotides, as expected.

The RNA transcripts were translated into ^{35}S-labeled polypeptides with Ambion's wheat germ IVT kit and approximately 45 μCi ^{35}S-methionine (Amersham; 37 TBq/mmol) in a 50-μl reaction. Labeled proteins were held on ice 6 hours prior to their use in chloroplast import experiments.

Pea (cv. "Little Marvel") and maize (inbred A188) plants were grown in a growth chamber at 25°C, 16 hour day length. Chloroplasts were isolated from pea and maize leaves 7 days after planting, respectively, as previously described (Burton et al., Pestic Biochem. and Physiol., 34, 76 (1989); Egli et al., Plant Physiol., 101, 499 (1993)). Intact mesophyll chloroplasts were washed in resuspension buffer [50 mM HEPES-KOH, pH 7.8 plus 0.33 M (pea) or 0.66M (maize) sorbitol] in preparation for import assays. Suspensions were diluted to obtain 75 μg chlorophyll/0.3 ml (Arnon, Plant Physiol., 24, 1 (1949)).

Import experiments were carried out essentially as described by Cline et al.(J. Biol. Chem., 260, 3691 (1985)). Import reactions containing 0.3 ml pea or maize chloroplast suspension, 40 μl ^{35}S-translation mixture, 3 mM Mg-ATP and 10 mM Met were incubated under light for 1-30 minutes at 25°C. Unimported proteins were digested for 30 minutes with 40 μg of thermolysin, and proteolysis was stopped with 10 mM EDTA.

Chloroplasts were re-isolated by centrifuging them through 1-ml 40% v/v Percoll gradients in the presence of resuspension buffer plus 3 mM Mg-ATP, 10 mM Met, and 20 mM EDTA, washed twice in the same buffer, and resuspended in 65 μl of 1 mM $MgCl_2$/10 mM Tris buffer, pH 8.0. Chloroplasts were lysed by three cycles of freeze-thawing in liquid N_2, microfuged, and

aliquots of the supernatants and of the original *in vitro*-translated proteins were analyzed by SDS-PAGE in 8-25% gradient Phast gels (Pharmacia), followed by direct detection of radiolabeled proteins in the wet gels (AMBIS) (Figure 20).

As estimated by SDS-PAGE, a 30 minute import converted the original 32-kD BCN1 polypeptide to a doublet of 27.2 and 27.5 kD in maize and produced an additional 30-kD band in pea (Figure 20A). Neither maize or pea chloroplasts imported -pBCN1-derived polypeptides. Formation of the 27.2-kD polypeptide likely resulted from cleavage after amino acid #47-49, a likely cleavage site because it lies between S- and D-rich regions, and R residues are located at -2, -7, and -8 (S. Gavel and G. Von Heijne, FEBS Lett., 261, 455 (1990)).

Time-dependence of BCN1 import was further examined (1-30 minutes) (Figure 20B) to determine if any imported polypeptides were a result of incomplete processing or proteolysis. Import was maximal after 15 minutes, but import time had no effect on the relative amounts of different-sized import products. Higher amounts of ATP (5 mM) stimulated import relative to lower amounts of ATP (< 0.2 mM). The data suggest that, in maize, efficient cleavage of BCN1 occurs at two closely adjacent sites and that partially processed products are also formed during BCN1 import by pea chloroplasts. Therefore, nucleotides 1-833 of the maize ACCase gene encode a CTP.

EXAMPLE IX

Expression of a cDNA Clone or Genomic Clones Encoding the ACCase Gene

The cDNA and genomic clones encoding all or a portion of the ACCase gene can be subcloned into a known expression system and the gene products reactive with the antibodies specific for maize ACCase can be identified using a Western blot. For example, the ACCase cDNA clones are inserted into two transformation plasmids: (i) Glb1exp which contains the embryo-specific maize Globulin*1* (*Glb1*) promoter and 3′ regions (Belanger et al., Genetics, 129, 863 (1991)); and (ii) pAHC17 which contains the maize ubiquitin (*Ubi-1*) constitutive promoter and first exon and intron, and the NOS 3′ terminator (Christensen et al., Plant Mol. Biol., 18, 675 (1992); Toki et al., Plant Physiol, 100, 1503 (1992)). The 3′ end of the A_1 cDNA has a unique *Sal*I site

just 3′ of the stop codon which is used to ligate into a *SalI* site in both plasmids ahead of the construct terminator. Other cloning sites will be added as needed to the plasmids or cDNA to complete the ligation of the 5′ end. The gene products can also be further characterized structurally and/or enzymatically. This will ensure that the genomic and cDNA clones that encode acetyl CoA carboxylase can be screened for promoters that provide for overproduction of the native or herbicide tolerant ACCase enzyme in plants.

For example, the 2 kb EcoRI fragment from clone #15-14 can be subcloned into a plant transformation plasmid pBI121 or pBI221 downstream from the 35S CaMV promoter and upstream from the nopaline 3′ polyadenylation signal sequence, as described in Jefferson, Plant Molec. Biol. Reptr., 5, 387-405 (1987). This plasmid can then be used to transform plant cells such as tobacco, Brassica and Arabidopsis cells using protoplast or biolistic transformation, as described by W.J. Gordon-Kamm et al., Plant Cell, 2, 603-618 (1990); M.E. Fromm et al., Bio/Technology, 8, 833-839 (1990); An, Methods in Enzymology, 153, 292 (1987); and D'Hafluin, The Plant Cell, 4, 1495 (1992). An increase in transient expression can be detected using quantitative Western blotting with antibodies specific for the ACCase enzymes. Polyclonal antibodies to maize ACCase most likely do not substantially crossreact with ACCase from dicots like tobacco or Arabidopsis.

Alternatively, the ACCase gene can be subcloned along with the 35S Came promoter into a binary Ti vector pGA482, as described in An, cited supra., which is a binary Ti vector system and can be used to transform plant cells by Agrobacterium-mediated transformation. Stably transformed plants can be generated by standard methods as described in Example III, and levels of expression of ACCase genes can be determined by quantitative Western blots, as described in Harlow and Lane, Antibodies, Cold Spring Harbor Laboratories (1988). The ability to monitor expression of cloned ACCase genes will permit the identification of promoters that provide for enhanced expression of the ACCase gene. The expression system can be used to screen for those promoters that enhance gene expression of the ACCase gene at least about 5 to 10-fold over the endogenous levels of ACCase produced normally in the plant cells. Because the 35S Came promoter is known as a strong promoter, it is likely this promoter

will provide for at least a 5-fold increase in the expression of ACCase over that normally produced in the plant cell.

In addition, this expression system can be used to screen antisense DNA sequences. For example, an antisense sequence can be obtained that is complementary to an about 0.5 kb region of the maize ACCase cDNA that has high homology with a portion of the chicken ACCase gene and contains the sequence for the presumed transcarboxylase active site domain, as shown in FIG. 8. The antisense sequence could be subcloned into a pBI121 or pBI221 expression under the control of an inducible plant promoter, such as nitrite reductase promoter (Back et al., Plant Molec. Biol., 17:9-18 (1991)). The ability of the antisense sequence to inhibit expression of the native ACCase gene can be evaluated in transformed cells, for example as described in Hamilton et al., Nature, 346:284-287 (1990).

EXAMPLE X

Identification and Cloning of the Gene From Herbicide Resistant Maize Cell Lines

Herbicide resistant maize cell lines were generated as described in Examples I, II, and IV. These herbicide resistant cell lines have been shown to produce an ACCase enzyme that is less sensitive to inhibition by sethoxydim or haloxyfop. The genes encoding the herbicide resistant forms of the ACCase will be identified and cloned using standard methods as described in Sambrook et al., Guide to Molecular Cloning: A Laboratory Manual (1989). The genes encoding the herbicide resistant forms of ACCase can then be introduced into herbicide sensitive plant species by standard methods to confer herbicide resistance . For example, the ACCase enzyme in the maize cell line 2167-9/2160-154 S-1 is at least 100-fold less sensitive to sethoxydim than the wild-type.

DNA from the cell line or plants will be obtained and digested with EcoRI and/or other appropriate restriction enzymes, according to standard methods. The restriction enzyme digest will be separated out by agarose gel electrophoresis and probed with either the 2 kb or the 3.9 kb cDNA ACCase probe described in Example V. Fragments hybridizing to the 2 kb or 3.9 kb probe will be subcloned into a Bluescript vector and portions of the gene will be

sequenced, as described in Example V, to verify that the entire ACCase gene has been isolated.

To confirm that the clone encodes the ACCase gene, it will be subcloned into the pBI121 or pBI221 expression vector, as described in Example VIII. The ACCase gene product expressed by the clone in either Black Mexican sweet corn cells or tobacco cells will be evaluated for reactivity with ACCase specific antibodies, by enzyme activity, and/or resistance of the enzyme activity to inhibition with sethoxydim and/or haloxyfop. It is likely that the cloned gene will encode an ACCase which is resistant to inhibition by sethoxydim and haloxyfop. This gene can then be introduced into an herbicide-sensitive embryogenic plant cell or an embryo, including maize cells or immature embryos, to confer herbicide resistance to that plant species upon regeneration.

The complete coding sequence encoding the herbicide resistant form of the ACCase enzyme will be cloned into a plant transformation vector such as pBI121 or pBI221 as described in Jefferson, Plant Molec. Biol. Reporter, 5:387-405 (1987). This vector contains the 35S Came constitutive promoter, the β-glucuronidase structural gene, and the nopaline synthase 3′ polyadenylation signals. The β-glucuronidase gene is replaced with a cloned ACCase gene. Optionally, the cloned ACCase gene can be combined with natural or synthetically produced chloroplast transit peptide sequence from pea, as described in Keegstra & Olsen, Ann. Rev. Plant. Physiol./Mol. Biol., 40:471-501 (1989) and/or unique restriction sites introduced so the cloned gene can be distinguished from the endogenous maize ACCase gene. Standard methods of subcloning will be utilized as described in Sambrook et al., cited supra.

For transformation of maize cells, type II calli can be transformed using biolistic transformation, as described by W.J. Gordon-Kamm et al., Plant Cell, 2, 603-618 (1980); M.E. Fromm et al., Bio/Technology, 8, 833-839 (1990); and D.A. Walters et al., Plant Molecular Biology, 18, 189-200 (1992). Alternatively, type I embryogenic calli can be transformed using electroporation after mechanically or enzymatically wounding calli, as described by D'Hafluin et al., The Plant Cell, 4:1495 (1992). Once the cloned gene is introduced into these cells and transformants are selected, typically by antibiotic resistance, fertile transgenic maize plants can be regenerated, as described by D'Hafluin et al. cited

supra. Fertile transgenic plants can be evaluated for herbicide tolerance, as described in Example III. It is likely that the fertile transgenic plants having and expressing a cloned ACCase gene as an ACCase resistant to sethoxydim and/or haloxyfop will exhibit herbicide tolerance as compared to the corresponding untransformed plant.

EXAMPLE XI
Generation of Transgenic Plants Having an Increase in Oil Content

Once identified and cloned, the gene or genes from maize acetyl CoA carboxylase can be introduced into monocot or dicot plant species, including maize, under the control of a promoter that provides for overexpression of the ACCase enzyme. The overexpression of the ACCase enzyme is likely to lead to an increase in the oil content of the plants and seeds.

Naturally occurring soybeans that have a high oil content and soybeans that have a low oil content have been identified. The acetyl CoA carboxylase from both types of soybeans was isolated, as described in Example V. The activity of the enzyme was measured as a function of the time of seed development and the results are shown in Figure 11.

The results in the Figure 11 indicate that higher oil content soybean is associated with a 2-fold increase in the ACCase activity during early to mid stages of development when compared with a low oil content soybean. Thus, increased expression of the ACCase gene correlates with an increase in the oil content of the seed. Total oil content of the seed was also measured at maturity (60 days). The high oil producing cell lines, Anoka and PI28C.134, have a total oil content of 21.8% and 19.9%, respectively. In contrast, the low oil soybean line of M76-395, has an oil content of 13.6% oil. Thus, the increase of ACCase expression early in seed development correlates with a higher total oil content in the seed at maturity.

A gene encoding a genomic maize acetyl CoA carboxylase can be isolated, as described in Example V, and used to transform plant species by protoplast or biolistic transformation. If the gene is combined with a strong promoter, such as the 35S cauliflower mosaic virus promoter, overexpression of the ACCase gene is likely. Alternatively, selecting transformed cells with multiple copies of the gene can also result in transformed cells overexpressing

the ACCase gene. The gene can be cloned into a vector such as pBI121 or pBI221, as described by Jefferson, cited supra. This vector contains the 35S cauliflower mosaic virus promoter, the β-glucuronidase structural gene, and the nopaline synthase 3′ polyadenylation signals. The cloned ACCase gene can replace the β-glucuronidase gene and then be used to transform plant cells, including maize, as described in Example VIII.

Transformed cells can be screened for overproduction of ACCase. The presence of the cloned gene can be verified by identifying the unique restriction enzyme sites incorporated into the cloned gene. ACCase levels can be assessed by standard enzyme assay methods and quantitative Western blots using antibodies specific for maize ACCase. Fatty acid and lipid content in cells lines overproducing ACCase are likely to be elevated and can be assessed using standard methodologies, as described in Clark & Snyder, JACS, 66:1316 (1989). Transformed cell lines overproducing ACCase and having increased total oil content will be used to regenerate fertile transgenic plants and seeds, as described in D'Hafluin, cited supra.

EXAMPLE XII

Generation of Transgenic Plants Having an Increase in Plastidic ACCase Activity

Maize embryos are transformed with sense and antisense cDNA constructs encoding the plastidic A1 ACCase. Selected transgenic cultures and regenerated transgenic plants and progeny are subjected to detailed analyses of: ACCase transcript levels; activity of ACCase I (plastidic) and ACCase II (presumably cytosolic) in various plant tissues; fatty acid synthesis; lipid accumulation in kernels (primarily embryos); and other plant traits. The culmination of these tests indicates whether plastidic ACCase activity can be modified via transformation and whether fatty acid synthesis is affected.

1. Transformation of maize embryos and plant regeneration

Immature maize embryos of the Hi-II genotype are transformed by particle bombardment according to slight modifications from previously described procedures (Fromm et al., Biotech., 8, 833 (1990); Koziel et al., Biotech., 11, 194 (1993)). This procedure has been employed in transformation studies for bombardment of approximately 15,000 embryos which were then

selected for Basta-resistant callus (*bar* selectable marker gene expression), and regenerated into plants. Transformed (Basta-resistant) plants are obtained from 1-2% of the initial embryos and, when separate plasmids are used for co-transformation, the nonselected transgene is recovered in about 50% of the Basta-resistant plants.

Basta-resistant, hemizygous transformed (T_0 = F1) plants will be tested by PCR or Southern blots for the presence of A1 ACCase sequences unique to the transformation vector, grown to maturity in growth chambers, greenhouse or field, and self-pollinated when possible, or backcrossed to a nontransformed parent. F2 or backcross progeny are grown in the greenhouse and field and tested for Basta resistance and presence of the A1 ACCase transgene to identify homozygous transgenic plants. Homozygous inbred transgenic lines are then developed.

2. Analysis of ACCase A1 transformants

Plants recovered from at least 100 independent transformation events (i.e., from different bombarded embryos) for both the UBI1 and GLB1 vectors are recovered. Regenerated plants are tested for the presence of the intact ACCase A1 transgene and its cosegregation with the Basta-resistance marker. Homozygous and heterozygous transgenic lines are assayed for total ACCase activity in leaves for UBI1 transformants and in developing embryos (22-26 DAP) for both UBI1 and GLB1 transformants. Sethoxydim and haloxyfop inhibition are used to quickly determine the levels of herbicide-sensitive ACCase I (plastidic) and the herbicide-insensitive ACCase II activity in both leaves and embryos. Increased expression of the ACCase A1 transgene contributes to plastidic ACCase activity and not to ACCase II activity. Kernel fatty acid and oil content are analyzed at maturity and their relationship with ACCase I determined by methods well known to the art.

Transformants that differ in kernel ACCase activity and/or fatty acid and oil content are then selected for more detailed analysis of embryos throughout development (4-day intervals from 16 DAP to maturity). These analyses include RNAse protection assays to determine total A1 + A2 transcript levels using a non-specific probe and to determine relative levels of endogenous A1 versus transgene A1 transcripts by use of antisense riboprobes spanning the

5′ UTR region of the A1 transgene constructs. Western blots of total proteins separated by SDS-PAGE gels are probed with the ACCase I-specific antibody described hereinabove or with avidin and analyzed by densitometry to distinguish changes in the 227-kD ACCase I and 219-kD ACCase II isoforms. ACCase I activity, fatty acid and lipid content are determined in embryos at each stage of development. These analyses determine whether expression of an additional gene(s) for plastidic ACCase increases ACCase activity and consequently fatty acid and oil content in maize tissues, especially in embryos.

3. Transformation with maize ACCase A1 antisense transformation vectors

Antisense transformation vectors were constructed by blunt-end ligation of nucleotides 1-833 of SEQ ID NO:5 in reverse orientation into multicloning sites of both the GLB1 and UBI1 plasmids. A sense construct with the same 833-bp cDNA sequence also was made with the GLB1 plasmid to serve as a transformation control. Insert orientations were verified by restriction mapping. UBI1 antisense, GLB1 antisense, and GLB1 sense constructs were introduced into > 2100, > 2900 and > 2000 embryos, respectively, and Basta-resistant callus were selected.

If antisense expression results in significant reduction in ACCase activity, it may not be possible to obtain viable callus or plants from the constitutive UBI1 antisense transformants. Similarly, plants transformed with the embryo-specific GLB1 antisense construct may exhibit deleterious effects on embryo development. Thus, failure to obtain transgenic progeny containing the antisense ACCase gene from these transformations may indicate that ACCase activity cannot be downregulated without loss of viability.

4. Analysis of ACCase A1 antisense transformants

All Basta-resistant cultures will be regenerated. The presence of the UBI1 and GLB1 antisense constructs will be determined by PCR analysis for unique transgene sequences such as the *Ubi-1* intron/ACCase A1 junction or *Glb1* 5′ UTR/ACCase A1 junction, or by Southern blotting to detect unique fragments. Plants and lines homozygous or heterozygous for the antisense transgene are analyzed for steady state level of the ACCase A1 antisense transcripts in appropriate tissues/organs (such as leaves, tassels, ears, embryos and endosperm for UBI1; leaves and embryos for GLB1) by using ACCase A1

sense riboprobes for hybridization on RNA blots or for RNAse protection assays. Total ACCase activity (both ACCase I and ACCase II isoforms) and fatty acid and lipid content are determined for the antisense transgenic lines and for corresponding tissues from nontransformed control plants. These analyses show whether ACCase A1 antisense transgenes are expressed in plants and, if so, whether expression is associated with reduced ACCase activity and altered fatty acid and lipid content in maize.

EXAMPLE XIII

Expression of Plastidic and Cytosolic ACCases during Plant Development

Intact embryos are isolated from developing kernels of field-grown inbred B73 at 2 to 4 day intervals between 16-42 DAP and frozen immediately in liquid nitrogen. Samples also are saved for fresh and dry weight determinations. Subsamples from each stage are analyzed for total lipid and fatty acid content. Seedling leaves are sampled along the leaf blade ranging from the etiolated, meristematic basal region to the fully expanded, green tip. Leaves and other tissues (e.g., epidermis) of maize genotypes that accumulate anthocyanin pigments are also analyzed to assess whether a specific ACCase (such as a cytosolic ACCase) is more highly expressed in tissues in which malonyl-CoA also is required as a substrate for chalcone synthase in the flavonoid pathway leading to anthocyanin synthesis.

Gene-specific antisense riboprobes in RNAse protection assays are employed to determine A1, A2, B1 and B2 transcript levels. The corresponding sense transcripts are produced *in vitro* and used as standards to verify specificity and quantitate the sample transcript levels. Quantitation is done on an AMBIS radioanalytic image system. Herbicide inhibition of total ACCase activity provides an assessment of levels of herbicide-sensitive ACCase I (plastidic) and the herbicide-insensitive ACCase II activity in these tissues. ACCase I and II isoforms are separated by ion-exchange chromatography. Total proteins are separated by SDS-PAGE and Western blots probed with avidin to detect the biotinylated 227-kD ACCase I and 219-kD ACCase II isoforms or probed with ACCase I-specific antibodies.

EXAMPLE XIV

Identification and Isolation of an Oat Gene Associated with Groat Oil Content

Cultivated oat, *Avena sativa* L. (2n=6x=42), is grown mostly in temperate regions. In many parts of the world, oat grain is produced for many purposes such as for animal feed, human food, and cosmetic and pharmaceutical additives (Webster, 1986). Seventy-eight percent of the world oat production is used for livestock feed (Schrickel, 1986). Among the cereals, oat groats have the highest lipid concentration (Morrison, 1978), but due to their high fiber content oat is lower in energy value than many other cereals when used for livestock feed. Oat is also unique among the cereals because more than 50% of total seed lipid is deposited in the starchy endosperm rather than the embryo of developing caryopses (Peterson and Wood, 1997; Youngs, 1986). An increase in oil content is desirable for making oat a higher-energy feed grain. On the other hand, a decrease in oil content would make oat a more attractive commodity for use in many current human diets where oat is desired for the type and concentration of its fiber (Ripsin et al., 1992).

A polygenic pattern of inheritance has been indicated for groat oil content with heritability estimates ranging from 63 to 93% (Schipper and Frey, 1991a) with primarily additive gene action (Thro and Frey, 1985). The average groat oil concentration in contemporary cultures is 6 to 7% but variation from 2 to 16% oil occurs in diverse germplasm (e.g., Kanota has about 11%, Ogle about 9% and Marion about 8% of oil (tryglycerides plus polar lipids) content, per dry weight). Genotype by environment interaction for this trait is small (Gullard, 1986; Branson and Frey, 1989). After five cycles of a recurrent selection program, Schipper and Frey (1991b) were able to produce oat lines with groat oil content greater than 16%. This response to selection is similar to the classical Illinois long-term selection experiments for oil content in maize (*Zea mays* L.) that resulted in dramatic progress toward altering the oil content in the original variety (Dudley et al., 1974). These studies demonstrate that, except when physiological limits are encountered, long-term selections for a quantitative, highly heritable and environmentally stable trait can prove successful. Analyses of 5987 oat samples representing a wide range of *Avena* germplasm showed a range of 2 to 12% for free non-polar lipids, with polar lipids contributing an

additional 2 to 3% (Youngs et al., 1982). Combinations of alleles for changing the groat oil content beyond this range may exist because this is mostly an unselected trait that exhibits large phenotypic diversity. Therefore, genetic dissection of this trait could expedite a program aimed at modifying the oil level of oat.

The recent explosion in development of restriction fragment length polymorphism (RFLP) linkage maps and their use in identifying genomic regions which affect quantitatively inherited traits is well documented (Phillips and Vasil, 1994). Molecular genetic studies relating to seed oil content have been reported for major oil-producing crop plants such as rape seed (*Brassica rapa* spp. *oleifera*; Tanhuanpaa et al., 1995a and 1995b), soybean (*Glycine max* L., Diers et al., 1992; Mansur et al., 1993; Brummer et al., 1997) and maize (Goldman et al., 1994; Alrefai et al., 1995). These studies indicate that genomic regions can be identified which significantly influence seed oil content. In the maize long-term Illinois selection material, two populations, the high oil by low oil population (Alrefai et al., 1995) and the high protein by low protein population (Goldman et al., 1994) were analyzed to identify genomic regions influencing kernel oil content. In both populations a region on chromosome 6 was identified near the linoleic acid1 locus. In the one case, this region explains 63% of the phenotypic variation in the ratio of oleic to linoleic (18:1 to 18:2) fatty acids and also influenced the total kernel oil concentration (Alrefai et al., 1995). To date, oat plants with an altered fatty acid composition as a result of a qualitative genetic mutation have not been identified, possibly due to the hexaploid nature of the genome.

In monocots, graminicides cause plant cell death by inhibiting ACCase and cessation of fatty acid biosynthesis (Burton et al., 1989). Maize mutants have been isolated that grow in the presence of normally lethal concentrations of ACCase-inhibiting herbicides, exhibit up to 20-fold increase in wild-type ACCase activity, and synthesize fatty acids in the presence of herbicide (Parker et al., 1990). Other results point to the role of ACCase in regulating seed oil deposition. A herbicide-insensitive ACCase mutation, Acc1, exhibits increased ACCase activity in corn embryos paralleling the reporting time course of oil deposition in this tissue (Somers et al., 1993). In developing

seeds of caster bean and rape, measured activity of ACCase correlates well with lipid accumulation providing additional evidence as to the role of this enzyme in fatty acid biosynthesis (Simcox et al., 1979; Turnham and Northcote, 1983). Moreover, expression of *Arabidopsis* cytosolic ACCase in *B. napus* plastids alters fatty acid composition, with the largest effect being an increase in oleic acid, and increasing total seed oil content by about 5% (Roesler et al., 1997). This increase in oleic acid is consistent with studies in maize indicating a positive correlation between percentage of oleic acid and total seed oil content (Alrefai et al., 1995).

Materials and Methods

Genetic Material

Two oat $F_{2:6}$-derived recombinant inbred populations developed by single seed descent (SSD) were used in this study: a population of 137 recombinant inbred lines (RILs) developed from the cross of a facultative winter-type cultivar, Kanota, by a spring cultivar, Ogle, (KO) and a second population of 139 RILs from the cross of Kanota by another spring cultivar, Marion, (KM). A portion (71 RILs) of the first population had been employed in previously reported oat mapping and QTL studies (O'Donoughue et al., 1995; Siripoonwiwat et al., 1996; Holland et al., 1997). The second population was chosen on the basis of having a common parent, Kanota, to provide a degree of "biological replication". These materials were grown in 1992-1995 in a randomized complete block design at the following locations;

Kanota X Ogle (KO):	Aberdeen, Idaho, 1992 and 1993; Ithaca, New York, 1993; and St. Paul and Rosemount, Minnesota, 1994.
Kanota X Marion (KM):	St. Paul and Rosemount, Minnesota, 1994; and St. Paul, Minnesota, 1995.

At Aberdeen, Idaho, the materials were grown in 3 replicates of four-row plots of 2.5 m in length, 30 cm between rows with the center 2 rows harvested. At Ithaca, New York, the materials were grown in 3 replicates of six-row plots of 4 m length and 18 cm between rows. At St. Paul and Rosemount, Minnesota, the materials were grown in 4 replicates of hill plots, with 30 seeds planted per hill

on 30 cm grid spacing. The exact parents used to generate the populations were included as single or multiple entries in each replicate.

Oil Content Analysis

To reduce the number of samples needed for analysis, equal amounts of sample from each replicate at a given location/year were bulked to represent a single balanced sampling per line. This resulted in five measurements for each RIL in the Kanota X Ogle population and three for the Kanota X Marion population.

Groat oil content was measured by a Bran+Luebbe InfraAlyzer 500 on dehulled ground samples. The Near Infrared (NIR) calibration was based on total fat as determined by acid hydrolysis. The calibration set contained 348 oat samples (representing commercial and elite breeding lines from North American oat breeding programs) with fat contents that ranged from 4.95 to 14.01%. The calibration equation had a correlation coefficient of 0.985, a Standard Error of Estimate of 0.299, and a F-value of 11500. Acid hydrolysis extracts the total lipid content of the groat. Thus, the reported values represent the sum of the neutral lipids (triglycerides) plus the polar lipid fractions (Youngs et al., 1982). Thus, these values are higher than those obtained from ether extracts which only contain the neutral lipid fraction.

Oat ACCase Clones or PCR Products

An oat groat cDNA clone was isolated based on its hybridization to a maize cDNA fragment which included portions of the less conserved mid-peptide sequence and the transcarboxylase domain (Shorrosh et al., 1994). Sequence identity indicated that the oat cDNA (3031 bp) corresponded to maize ACCase nt 3124-6169 (Egli et al., 1995). The 1010-aa predicted polypeptide was nearly identical to portions of wheat (89%) and maize (84%) plastidic multifunctional ACCases; it had lower identity to cytosolic ACCases, including wheat (67.5%) and alfalfa (64.6%) (Gornicki et al., 1994; Shorrosh et al., 1994).

An amplified 'Prairie' oat developing groat cDNA library (R. Skadsen, USDA, Madison, WI) cloned into the EcoRI site of λZAPII (Stratagene) was screened with a [^{32}P-dCTP]-labelled maize ACCase cDNA probe (Ausabel et al., 1997) derived from an EcoRI digest of clone 18-5 (nt 2020-5927; Egli et al., 1995). Plaques from eleven partially purified positives

were eluted in SM medium (Sambrook et al., 1989) and excised *in vivo* to obtain pBlueScript plasmids carrying oat cDNA inserts, according to the manufacturer's instructions (Stratagene Corp.). Five colonies per transformation reaction were screened (as colony lifts) with an AflII-BbrPI fragment of maize ACCase cDNA (4281-5797; Egli et al., 1995). One clone, oat-91, hybridized to the probe. Oat-91 was sequenced using the ABI Prizm system (Perkin-Elmer Corp.) at the Advanced Genetic Analysis Center (Univ. of Minnesota, St. Paul, MN). Sequence identity indicated the oat-91 EcoRI insert corresponded to maize ACCase nt 3124-6169 (Egli et al., 1995). Oat ACCase cDNA sequence was compared to other plant ACCases with the BESTFIT program (BESTFIT, Wisconsin Package V. 9.1, Genetics Computer Group (GCG), Madison, WI).

For PCR, Pileup alignments of maize, wheat, barley, and rice ACCase coding sequence (GCG, Madison, WI; 12, 18, F, R) were used to identify conserved regions (i) for design of new primers or (ii) regions of identity to available maize ACCase oligos, to be used for PCR amplification of oat ACCase. Two maize primers, PCR2 (5′-ggcagagcaa agcttggagg; SEQ ID NO:25) and PCR1 (5′-tccaccagag aagcctctcc; SEQ ID NO:26) appeared like to amplify oat cDNA based on their identity to other monocots and previous use to amplify soybean ACCase. These primers are located within the transcarboxylase domain, surrounding the acetyl-CoA binding site of ACCase. An amplified 'Prairie' oat seedling leaf cDNA library cloned into the EcoRI site of IZAPII (Skadsen, University of Wisconsin) was used as a source of cDNA. One I of the oat cDNA library was amplified with maize primers (PCR2/PCR1), using the Expand High Fidelity PCR system (BMB) in a GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer) after denaturing phage heads at 94°C for 10 minutes. The single, 222-bp product was purified by Wizard PCR Plus (Promega Corp.) and sequenced in an ABI377 (Perkin-Elmer) using PCR1 as primer. The resulting sequence appeared to be from oat since it had high identity to maize plastidic ACCase (89%). Additional 5′ cDNA was obtained by (i) PCR of the oat cDNA library with T3/PCR1 primers to generate a complex band mixture and (ii) reamplification of 0.1 I products with maize ACCase primers 1324+ (5′-caatcacgtcagactg; SEQ ID NO:27)/PCR1 to obtain a 1.9-kb product with the same 3′ sequence as the PCR2/PCR1 product.

RFLP and Statistical Analysis

Briefly, two statistical procedures were used to determine the location, effect and genotype by environment interaction (GxE) for significant QTLs. The first procedure was single factor ANOVA (ANOVA and GLM procedures; SAS Institute, 1990) with the added criteria for consistency in association of genomic regions with a trait over environments and linkage distances (Kianian et al., 1998). If the association were significant when considering the average of all the environments, the association was also significant over the majority of the environments (3 of 5 in KO and 2 of 3 in KM), and closely linked markers within that region, if available, also showed significant associations. A "backward elimination" process as described by Kennard and Havey (1995) was used to construct a multi-locus model containing the most important markers identified by the ANOVA procedure. Marker loci were excluded one at a time from this model, based on the criterion of least significant ($P < 0.05$) Type-III sum of squares (Kennard et al., 1994). In addition, MQTL software (Tinker and Mather, 1995a & b) was used to strengthen and validate the ANOVA results. This software performs Simple Interval Mapping (SIM) and Simplified Composite Interval Mapping (sCIM). It also has the advantages of searching for one QTL while simultaneously accounting for the effect of others, providing separate analyses for each environment, and calculation of a threshold to control the type-I error rate. One thousand permutations were performed to estimate the threshold for a type-I error rate below 5%. Significant QTL regions were determined as described by Tinker et al., 1996. These regions were grouped in a multi-locus linear model to estimate the overall phenotypic variance explained by the model. However, additional criteria were used to include markers in the final MQTL model, which gave 10 cM spacing between QTLs, and minimized the number of regions excluding those markers explaining less than 5% of the phenotypic variance, while maintaining most of the overall R^2.

Results

Trait Data

Groat oil concentration for both populations showed a relatively continuous distribution with many of the individuals having values within one standard deviation of the parents (Table VI). Among the three parents, Kanota showed the highest concentration of groat oil content followed by Marion and then Ogle. The groat oil content value for the Kanota line derived from the parent used in generating the KM population was higher than that used for the KO population (Table VI). This is not unexpected: Kanota is a heterogeneous population that shows variation for other traits, but individual parent lines were used in population development and were maintained as controls for each population. Large numbers of individuals having values outside the range of parents were observed for both populations (Table VI). Transgressive segregants tended more toward the lower groat oil concentration than the high. The KO population contained individuals with the highest and lowest groat oil concentrations, possibly due to a larger difference in parental values (3% between Kanota and Ogle, 2% between Kanota and Marion). Overall, the KM population contained more transgressive segregants than the KO population (Table VI).

Table VI. Groat oil concentration and percent transgressive segregants in two recombinant inbred oat populations

Population (P1 × P2)	Location	Parents		Population				% Transgressive segregants			
		P1	P2	Mean	SD	Min.	Max.	X<P2	X>P1	X<P2-SD	X>P1+SD
Kanota X Ogle	Aberdeen 92	11.5	8.3	9.7	1.3	7.5	14.0	13	8	0	2
	Aberdeen 93	10.5	8.4	10.1	1.6	7.2	15.4	18	35	0	8
	Ithaca 93	10.9	7.1	9.0	1.4	6.5	13.6	8	8	0	2
	St. Paul 94	10.9	7.7	9.2	1.4	6.6	13.4	13	10	0	2
	Rosemount 94	11.1	8.4	9.0	1.3	6.7	12.6	30	5	5	1
	Average	11.0	8.0	9.4	1.4	6.9	13.8	16	13	1	3
Kanota X Marion	St. Paul 94	12.9	10.2	10.2	1.0	8.0	13.3	47	1	12	0
	Rosemount 94	12.2[a]	9.2	10.4	1.2	7.5	14.2	10	7	1	3
	St. Paul 95	10.0	8.4	8.8	0.7	7.1	10.8	26	2	4	1
	Average	11.5	9.3	9.8	1.0	7.5	12.8	28	3	6	1

[a] Adjusted average value for the parents calculated based on the mean of population at that location divided by the overall population mean times the average parental values was used in this calculation (i.e., (10.4/9.8)11.5).

SD = Standard Deviation

Table VII. Phenotypic correlations for groat oil concentration between environments*

Population	Location	Aberdeen 92	Aberdeen 93	Ithaca 93	St. Paul 94
Kanota X Ogle	Aberdeen 93	0.94			
	Ithaca 93	0.94	0.97		
	St. Paul 94	0.90	0.91	0.92	
	Rosemount 94	0.91	0.95	0.96	0.94

		St. Paul 94	Rosemount 94
Kanota X Marion	Rosemount 94	0.87	
	St. Paul 95	0.80	0.71

* All values are considered significant at $P < 0.0001$.

The correlation among the different environments for oil content was high (Table VII). Measurements for the KO population had an average R of 0.93 with the lowest value of 0.90 between material grown at Aberdeen, ID 1992 and St. Paul, MN 1994 ($P < 0.0001$). Values for the KM population were less well correlated (average R of 0.79) with the lowest correlation between the samples grown in Rosemount, MN 1994 and St. Paul, MN 1995 ($P < 0.0001$). The difference in the size of the population correlations could be due to a smaller range of values for the KM population (5%) as opposed to the KO population (7%), or fewer measurements (3 vs. 5 environments), or both.

Kanota X Ogle Population

RFLP Data

A hexaploid oat RFLP map that contains 561 loci has been generated from 71 RILs in the KO population (O'Donoughue et al., 1995). Initial quantitative analysis was performed using this subset of the KO population, the available trait data, and 360 of the 561 loci that are co-dominantly segregating and evenly distributed across the oat genome. To take advantage of the entire population and to add power to the analysis, the remaining 66 RILs in this population were also mapped. To date, 153 loci have been mapped with these individuals. Detection of QTL with either the subset (360 markers and 71 individuals) or the entire data set (153 markers and 137 individuals) of the KO population did not make a major difference, except in two situations. In both cases, QTLs were detected in the subset, but were not detected in the entire population due to the absence of RFLP data for the remaining 66 RILs. Results of the entire population are presented herein except for the aforementioned differences.

An RFLP map containing all 137 individuals and the 153 mapped loci was generated and was in accord with the published map (O'Donoughue et al., 1995) except for linkage group 3. An inter-varietal translocation is believed to involve this linkage group. In the final analysis, the published map was used for this region. Additional information from aneuploid analysis (Kianian et al., 1997) was also taken into account when two linkage groups were assigned to the same chromosome. Thus, linkage groups 5 and 7 assigned to chromosome 5C, linkage groups 6 and 20 assigned to chromosome 16, and linkage groups 4 and

12 assigned to chromosome 21, were analyzed as three distinct units. In order to have a complete coverage of the genome for SIM and sCIM, 344 mapped loci (191 loci scored on only 71 RILs) were used in the MQTL analysis.

Quantitative Analysis

Analysis of variance using the criteria mentioned revealed eight linkage groups and one unlinked marker significantly associated with groat oil content ($P < 0.05$ for individual environment and $P < 0.0001$ using means across environments). These regions are on linkage groups 6 and 7 (Table VIII). Two additional genomic segments on linkage group 37 and an unlinked locus *Coleoptal* were significant in tests of the first 71 individuals of the mapping population but could not be verified on the whole population due to a lack of RFLP data for the entire set. The positive alleles (those that cause an increase in groat oil content) for all of the genomic regions influencing this trait were from the Kanota parent (Table 3). Overall, the alleles from Kanota contributed 2.8% (4.1% if linkage group 37 and *Coleoptal* loci were included) to the groat oil content. Regions on linkage groups 6 and 11 are considered highly significant and remained in the full model after the backward elimination process (Table 4). However, the two additional loci from linkage group 37 and *Coleoptal* were also included in the model if only the first 71 individuals in this population were being analyzed. However, the two additional loci from linkage groups 37 and *Coleoptal* were also included in the model if only the first 71 individuals in this population were being analyzed. Locus Xcdo665B on linkage group 11 accounted for 36% to 48% (depending on the environment) of the phenotypic variance for this trait ($P < 0.0001$). For these linked loci, a substitution of Kanota alleles for their Ogle counterparts caused an average increase of 2% in groat oil content. Substitution of Kanota alleles for Ogle alleles at the locus *Xcdo1414B* (or *Xumn51B*) on linkage group 37 caused an average increase of 1% in groat oil content and was the second most significant locus considering only the first 71 individuals in this population.

Table VIII. Genomic regions significantly influencing groat oil content in oil identified by single factor analysis of variance

Kanota X Ogle					Kanota X Marion				
Linkage Group	Locus[a]	Source	R^2	% Oil[b]	Linkage Group	Locus[a]	Source	R^2	% Oil[b]
6	*Xcdo1357C*	Kanota	0.10	0.8	3	*Xbcd1562A*	Kanota	0.05	0.4
11	*Xcdo665B*	Kanota	0.38	2.0	11	*Xcdo665B*	Kanota	0.18	0.7
37[c]	*Xcdo1414B*	Kanota	0.10	1.0	22	*Xumn5004*	Kanota	0.05	0.3
Unlinked[c]	*Coleoptal*	Kanota	0.13	0.3	5X[d]	*Xcdo1199 C*	Kanota	0.11	0.7
Total				2.8 (4.1[b])					2.1

[a] Locus with the highest R^2.

[b] Substitution of the source alleles will cause on average (based on average of values from each of five environments in KO and three in KM) this change in groat oil concentration. $P < 0.0001$ for the mean across all environments and $P < 0.05$ for each individual environment.

[c] Significant region identified only in the first 71 individuals of this population.

[d] More markers are needed to accurately place this linkage group on the hexaploid oat RFLP map.

Table IX. Multi-locus models for groat oil concentration in oat

Population	ANOVA Model[a]	MQTL Model[b]
Kanota X Ogle	*Xcdo1357C* (6, K)	*Xcdo665B* (11, K)[c]
	Kcdo665B (11, K)[c]	*Xcdo1414B* (37, K)
	Xcdo1414B (37, K)[d]	*Coleoptal* (Unlinked, K)
	Coleoptal (Unlinked, K)[d]	
Total R^2	**0.45 (0.67)[e]**	**0.47**
Kanota X Marion	*Xcdo665B* (11, K)[c]	*Xcdo665B* (11, K)[c]
	Xumn5004 (22, K)	*Xumn5004* (22, K)
	Xcdo1199C (5X, K)	*Xcdo1199C* (5X, K)
Total R^2	**0.28**	**0.20**

[a] The linkage group and parent contributing the positive allele are depicted in parentheses (i.e., linkage group 11 with Kanota allele being positive (11, K)). The models are based on five environments in KO and three in KM.

[b] The VM/VP (variance explained by the QTL(s) over the phenotypic variance) is presented as the R^2 for MQTL.

[c] *XaccaseA* is closely linked to *Xcdo665B* and gives the same relative effects as *Xcdo665B*.

[d] These additional loci fit the model when the analysis included only the first 71 individuals in this population.

[e] Total R^2 with markers marked d is presented in parentheses.

Quantitative analysis using the MQTL software gave a slightly different result. Like the ANOVA results, a highly significant locus ($P < 0.0001$), Xcdo665B on linkage group 11, was identified at a test statistic (TS) value of 290.2 that is roughly equal to a LOD score of 64 (0.22 × TS). This locus overshadowed any other significant region and accounted for more than 37% of the phenotypic variance [Variance QTL Main effect (VM)/Variance Phenotypic (VP) = 38%, Variance QTL × environment Interaction and VM (VI)/VP = 38% and Variance Genetic from background markers (VG)/VP = 7%)] and a change of 2% in groat oil content (Figure 23). However, secondary loci that showed significance below the threshold value (threshold TS = 58.2 after 1000 permutations) were inferred when either SIM or sCIM gave evidence for QTLs. Xumn51B on linkage group 37 and the Coleoptal locus, which is unlinked, were significant at test statistic values of 49.9 and 44.8 respectively. The three loci together accounted for 47% of the phenotypic variance and a change of 3.3% in groat oil content (VM/VP = 47%, VI/VP = 48% and VG/VP = 7%). For all three loci the Kanota parent contributed the positive allele. These loci showed a remarkable consistency over the five environments. Thus, there were no significant peaks inferred from the QTL × E interaction scan (Figure 23).

Kanota X Marion Population

RFLP Data

The results from an initial quantitative analysis of the KO population (first 71 RILs) for groat physical and chemical traits were used to determine markers for mapping the KM population. The markers mapped were not chosen for their association with groat oil content in the KO subset population, but for their association with other groat chemical and physical characteristics (Kianian et al., 1998). Since the level of polymorphism in hexaploid oat is relatively high, most probes detected sequences mapping to more than one location. A total of 60 loci were mapped with the 139 RILs in the KM population. These markers mapped to 20 of the 38 linkage groups that had been identified in the KO population (linkage groups 1, 2, 4, 5, 8, 9, 18, 19, 21, 24, 25, 26, 30, 33, 34, 35, 36, 37, 38 were not covered). The linkage groups not covered were, in general, the smaller ones consisting of only two to a few

markers in the KO map. The data from the KM population generated an additional linkage group of two loci, and 13 markers remained unlinked. Except for linkage group 37 and *Coleoptal* locus, this KM map covered all regions with significant associations with groat oil concentration in the KO population.

Quantitative Analysis

Single factor ANOVA revealed four linkage groups significantly associated with groat oil content in the KM population. These were groups 3, 11, 22, and 5X ($P < 0.05$ for individual environment and $P < 0.0001$ using means across environments). As in KO, all of the genomic regions influencing this trait were from the Kanota parent. Overall, the Kanota genomic regions contributed 2.1% to the groat oil content. Regions on linkage groups 11, 22, and 5X (group 5X has been tentatively assigned to linkage group 5 but more markers are needed to accurately place this linkage group on the hexaploid oat RFLP map) are considered highly significant and remained in the full model after the backward elimination process. As in the KO population, locus *Xcdo665B* on linkage group 11 was highly significant for its effect on groat oil content ($P < 0.0001$), accounting for 16% to 20% (depending on the environment) of the phenotypic variance for this trait. Overall, the three significant loci explained 15% of the phenotypic variance and together accounted for a total difference of 1.7% in groat oil content.

Analysis by SIM or sCIM gave the same result as ANOVA. Linkage groups 11, 22, and 5X were significant in the KM population. Locus *Xcdo665B* on linkage group 11 was significant at a test statistic value of 70.5 (LOD = 15.5). This locus was not as dominant in the KM population as it was in the KO population. This locus alone explained 18% of the phenotypic variance (VM/VP = 18%, VI/VP = 19% and VG/VP = 3%) and accounted for 0.9% change in groat oil content. The other significant locus above the threshold value was *Xcdo1199C* on linkage group 5X (TS = 33.1) accounting for a change of 0.2% in groat oil concentration. A secondary locus inferred was *Xcdo506B* on linkage group 22, which accounted for 0.4% change in oil content. These three loci together accounted for 20% of the phenotypic variance and 1.5% in groat oil content (VM/VP = 20%, VI/VP = 22% and VG/VP = 3%). As expected from the trait data, these loci showed a varied effect over the three environments and one

interaction on linkage group 11 is notable (Figure 23). The QTL × E interaction peak on linkage group 11 was at TS = 6.6, slightly below the significant threshold, and was due to a range of 0.5% in groat oil content explained [the values for the groat oil content were 0.6% (St. Paul, Minnesota, 1995) and 1.1% (Rosemount, Minnesota, 1994)].

ACCase and its Relationship to Groat Oil Content QTLs

An oat groat cDNA was isolated based on its hybridization to a maize cDNA fragment which included portions of the less conserved mid-peptide sequence and the transcarboxylase domain (Shorrosh et al., 1994). The 3031-bp oat cDNA was nearly identical to wheat (90%) and maize (84%) genes encoding plastidic ACCase (Egli et al., 1995; Gornick et al., 1997). A 424 nucleotide sequence form the 3′ end of the insert was translated and compared to other plant ACCase peptides. The oat peptide sequence was 93% identical to plastidic isozymes from maize and wheat (Egli et al., 1995; Gornicki et al., 1997), but only 78 to 83% identical to cytosolic ACCase from maize, wheat (Gronicki et al., 1994), or alfalfa (Shorrosh et al., 1994).

The 3031-bp oat ACCase cDNA clone identified a single polymorphic locus in the KO population (*AccaseA*) and two polymorphic loci in the KM population (*AccaseA* and *AccaseB*). The *AccaseA* locus maps 2.3 cM from *Xcdo665B* and 3.7 cM from *Xisu2287* on linkage group 11. The *AccaseB* locus maps 11 cM from *Xcdo665D* and 7.3 cM from *Xbcd1729B*. In the KO population *AccaseA* explains on average 37% of the phenotypic variance and in KM 18% of the phenotypic variance for groat oil content (for both cases $P < 0.0001$ using means across all environments). The individuals in both populations can be divided into two groups based on their genotype for this locus and groat oil content using the mean across all environments (Figure 3). These two classes differ in their oil content by an average of 1.67% in KO and 0.78% in KM. Results of QTL analysis for the *AccaseA* locus are the same as that reported for *Xcdo665B* (Table 4).

Table X. Peptide sequence identity of oat and other multifunctional ACCases. Partial, internal sequences are 1010 amino acids unless noted otherwise. Presence of an N-terminal plastid transit peptide is noted, if known.

ACCase Gene source	GenBank #	Transit Peptide?	% identity
Avena sativa	AF072737	?	100
Triticum aestivum	AF029895	yes	89.2
Zea mays	U19183	yes	84.1
Triticum aestivum	U10187	no	67.5
Zea mays	Egli, unpubl.	?	78.6 #
Hordeum vulgare	X99102	?	71.9 §
Arabidopsis thaliana	L27074	no	63.7
Brassica napus	X77576	yes	63.9

based on 145 aa at the C-terminus of oat-91
§ based on 498 aa at the C-terminus of oat-91
? partial cDNA, unknown N-terminus

Discussion

The ACCase cDNA clone from developing oat seeds hybridizes to a genomic region that is linked to a QTL locus on linkage group 11 having a major influence on the total lipid content of oat groats. The influence of this locus was detected in various environments and two genetic backgrounds, thus confirming the value of biological as well as environmental replication. These results provide strong support for the hypothesis that ACCase has a major role in determining groat oil content in crop plants. Either allelic variation in ACCase activity controls oil content in oat groats, or a gene(s) closely linked to ACCase is responsible. In other studies of oat directed at transferring a major crown rust resistance locus on linkage group 11 (Bush et al., 1994), lines with the introduced rust resistance gene often had altered groat oil content. These results further indicate the likely presence on linkage group 11 of a genetic locus with a critical role in the fatty acid biosynthetic pathway in oat. Mutations, which cause a gradual but demonstrable major effect on a biochemical pathway, are believed to be the most common type in polyploids (Sears, 1972).

Identification of a major quantitative trait locus influencing the biochemical composition of grain is not unusual. Studies of fatty acid, starch, and protein concentrations and composition provide good examples (Goldman et al., 1993 and 1994; Alrefai et al., 1995; Hu et al., 1995). In many of these cases,

the significant region is in close proximity to a known genetic locus with a demonstrated effect on the trait of interest. For instance, in maize, a major genomic region for fatty acid concentration and composition has been identified near the *linoleic acid-1* locus, and one influencing protein and starch concentration was identified near the *Shrunken-2* locus (Goldman et al., 1993 and 1994; Alrefai et al., 1995). These and numerous other reports bolster the hypothesis that allelic variants at known genetic loci may be responsible for quantitative effects (Robertson, 1985).

Reliable identification of genomic regions with an influence on a particular quantitative trait has always been difficult. In a number of studies conducted on a given quantitatively inherited trait in various populations and environments, the most significant regions tended to be in common (Goldman et al., 1994; Alrefai et al., 1995). However, the accuracy of identifying a quantitative trait locus will depend, in part on the degree to which it influences the trait in a given population. This is demonstrated in our study by the locus on linkage group 11 which has similar but varied effects in different populations. Marion has a higher concentration of groat oil than Ogle and more loci with positive influence on this trait. The locus on linkage group 11 increased the groat oil content to a lesser degree in the KM population than in the KO population. Correspondingly, the relative proportion of variance due to the environmental influence on this locus was much higher in the KM population than in the KO population. This example illustrates the power of biological replication (i.e., having populations with a common parent) in identifying quantitative trait loci.

Identification of the most significant locus does not preclude the identification of other less important loci due to the influence of genetic background and environment on the overall effect. The consistency in identification of significant regions by various quantitative analysis methods in the KM population is a good example. The regions on linkage groups 5, 11 and 22 and Xcdo395C locus are considered to make important contributions to the groat oil content in this population. The Xcdo1199C locus located near the Xacor242 locus on linkage group 5 of the published hexaploid oat RFLP map (O'Donoughue et al., 1995) has as great an effect as Xcdo665B-XaccaseA region

on linkage group 11 in this population. This locus was not detected in the KO population presumably because the Ogle allele had a similar influence as Kanota and in the KM population the Marion parent was carrying the allele with positive influence on the groat oil content. The other two significant loci in the KM population have a relatively minor effect on this trait and would be more difficult to detect in a population segregating for regions with a larger influence on oil such as in the KO population.

Results from other QTL studies can help identify possible pleiotropic effects of other traits such as heading date and vernalization response on groat oil concentration. A region on linkage group 3 with alleles being contributed by Kanota is implicated as having a major influence on heading date and possibly yield, thus reducing its importance as having a direct effect on groat oil concentration (Siripoonwiwat et al., 1996; Holland et al., 1997). The cultivar Ogle was found to have loci on linkage groups 22 and 37 that influence plant height in the KO population (Siripoonwiwat et al., 1996). Loci on linkage group 11 is believed to have an influence on test weight and other groat physical characteristics, thus providing additional evidence of their role in oat groat morphophysiology (Siripoonwiwat et al., 1996; Cakir et al., 1996). These and other studies of quantitative traits in oat are helping identify genomic regions that influence the growth, development and physiology of grain, the primary product. Thus, genetic and molecular manipulation of the oat genome to improve grain quality and quantity can be directed at critical segments.

Cultivated oat is a hexaploid composed of three ancestral genomes designated A, C, and D (Rajhathy and Thomas, 1974). Recent studies have indicated that the organization of hexaploid oat genomes into discrete homoeologous groupings such as that of hexaploid wheat is difficult (Rooney et al., 1994; O'Donoughue et al., 1995; Kianian et al., 1997). However, C-genome chromosomes, due to their distinctiveness, have been used as a basis to identify homoeologous segments among the other chromosomes (Kianian et al., 1997). Thus, it is believed that a type of segmental homology instead of whole chromosome homology best describes the genome organization in hexaploid oat (Kianian et al., 1997). Association of homoeologous genomic segments with a quantitative trait further substantiates that relationship.

The region marked by the probe CDO1414 can identify both the significant region on linkage group 11 and the one on linkage group 37 indicating possible homoeology of these regions. The same is true for probes CDO1199 for linkage groups 6 and 5X and CDO708 for linkage groups 6 and 22. Thus, the association of groat oil content with RFLP markers in oat is explained by at least three sets of homoeologous genomic segments.

In hexaploid wheat plastidic ACCase maps to the short arm of homoeologous group 2 near the telomere (Gornicki et al., 1997). Plastidic ACCase genes in wheat have been shown to be transcriptionally active in seedling leaves but their effect on seed lipid content are unknown (Gornicki et al., 1997). ACCase in maize has been mapped to short arm of chromosome 2 near the centromere and long arm of chromosome 10 (Egli et al., 1995; Van Dee, 1994). A QTL with moderate effect on oil concentration in progeny of Illinois High and Low oil maize lines was located near the ACCase locus on maize chromosome 2 (Berke and Rocheford, 1995). Additionally, there are numerous *defective kernel (dek), reduced endosperm (ren)*, and *Endosperm factor (Ef)* loci influencing grain morphophysiology located on chromosomes 2 and 10 of maize near these synthetic segments (MaizeDB). In maize, oil is stored in embryos and embryo size is associated with oil content of whole seeds (Dudley, 1974). Unlike maize, oat grains contain significant amounts of lipid in the endosperm (Peterson and Wood, 1997), and thus QTLs affecting pathway flux could be more important than those altering seed anatomy. The regions in wheat and maize agree well in terms of conserved syntony and correspond to chromosome 4 of rice, linkage group C of diploid oat and linkage group 11 of hexaploid oat map (Ahn and Tanksley, 1993; Van Deynze et al., 1995). This comparative evidence from other grass species and homoeologous evidence from oat further help reinforce the validity of the association of these genomic regions with groat oil content and composition in oat.

Summary

Oat groats are unique among cereals for the high level and the embryo-plus-endosperm localization of lipids. Genetic manipulation of groat quality traits such as oil is desired for optimizing the value of oat in human and

livestock diets. A locus having a major effect on oil content in oat (*Avena sativa* L.) groats was located on linkage group 11 by single factor analysis of variance, simple interval mapping and simplified composite interval mapping. A partial oat acetyl-CoA carboxylase (ACCase) cDNA clone with >84% identity to genes for plastidic ACCase of maize and wheat, which catalyzes the first committed step in *de novo* fatty acid synthesis, identified a polymorphism linked to this major locus. Similar QTL and ACCase locus placements were obtained with two recombinant inbred populations having one parent in common (Kanota X Ogle and Kanota X Marion) and containing 137 and 139 individual lines, respectively. By having a common parent, these populations provide biological replication of the results in that significant genomic regions should be evident in analysis of multiple cross combinations.

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In contrast to oat, a maize ACCase gene was linked only to a moderate oil QTL. Unlike maize, where embryo size affects seed lipid concentration, oat stores significant amounts of lipid in the endosperm, thus QTLs affecting pathway flux may be more important than those altering seed anatomy. If an ACCase gene is indeed the major groat oil QTL, differential ACCase gene expression and/or enzyme activity may control groat oil content.

Moreover, if an ACCase DNA is a QTL in oat, it may be useful as a marker for marker-assisted breeding in oat and other cereals, e.g., maize and wheat.

Table XI. Genomic regions significantly influencing groat oil content in oat. Groat oil values were averages of five (KXO) or three (KxM) environments.

Kanota x Ogle

Locus	Linkage Group	High-Oil Parent	Coefficient of variation	Groat oil, % dry wt
Xcdo665B	11	K	0.38	2.0 #
XaccaseA	11	K	0.38	2.0
Coleoptal	unlinked	K	0.13	0.3
Xcdo1414B	37	K	0.10	1.0
Xcdo1357C	6	K	0.10	0.8
				4.1 Total

Kanota x Marion

Locus	Linkage Group	High-Oil Parent	Coefficient of variation	Groat oil, % dry wt
Xcdo665B	11	K	0.18	0.70 #
XaccaseA	11	K	0.18	0.70
Xcdo1199C	5X	K	0.11	0.70
Xbcd1562A	3	K	0.05	0.40
				1.80 Total

Xcdo665B and *XaccaseA* were associated with the same QTL.
Groat oil values were averages of five (KxO) or three (KxM) environments.
QTLs were identified by single factor analysis of variance.

EXAMPLE XV

Method to Decrease Oil Content in Oat Seed

Youngs (1986) reviewed 11 studies reporting lipid content of oat groats and found an overall range of 2.0 to 11.8% oil concentration in oat groats. Contemporary oat cultivars exhibit an average of 6 to 7% oil (1994-96 Midwest Regional Uniform Nursery data). Recently, Peterson and Wood (1997) reported on the composition and structure of high-oil oat selections ranging from 6.9 to 18.1% oil. While this level of lipid is beneficial for animal feeding because high

oil is associated with higher energy value in the feed, high oil content in oats causes rancidity problems during processing. Rapid liberation of fatty acids from triglycerides by endogenous lipases results in fatty acid oxidation. Furthermore, use of current oat cultivars results in relatively high-fat food products, e.g., oatmeal. Oat processors indicate that a 50% reduction of groat lipid content, while maintaining the superior agronomic characteristics of the currently existing cultivars, would be highly desirable.

Oat is unique among the cereals in regard to seed lipid deposition (Welch, 1995; Youngs, 1986). Most other cereals accumulate storage lipid primarily in the embryo of developing caryopses. Mature oat groats contain more than 50% of total seed lipid content in the starchy endosperm (Peterson and Wood, 1997). Additionally, significant amounts of oil are found in the bran fraction of processed oats. A substantial portion of the bran is in starchy endosperm and aleurone cell layers which do not separate cleanly from the bran as in wheat. Together the oat endosperm and bran fractions contain >90% of the total lipid in the groat. Thus, the endosperm is the major source of the oil in the groat.

Two distinct types of ACCase are known. The first type is typical of eukaryotic organisms where a single, nuclear gene encodes the biotin carboxylase, biotin carboxyl carrier protein, and carboxyl transferase domains within a single, multifunctional polypeptide of 210-280 kDa that, in plants, forms a dimeric active enzyme. Plant lipid synthesis begins in plastids, where ACCase synthesizes the malonyl-CoA needed to initiate and elongate fatty acids of up to 18 carbons (Guenther, 1996). To date, multifunctional ACCase genes from maize, wheat and *Brassica* have been shown to encode functional chloroplast transit peptides. Both monocots and dicots also appear to contain a cytoplasmic form of the multifunctional ACCase. The cytoplasmic ACCase may be involved in the synthesis of malonyl-CoA needed for such reactions as the synthesis of very long chain fatty acids and flavonoids in plants.

The second, multisubunit type of ACCase consists of three functional domains formed from four separable polypeptides and is found in dicot plastids and in prokaryotic organisms, but appears not to be present in the *Gramineae*. The biotinylated polypeptide component of this multisubunit form

of ACCase is absent from the *Gramineae* and the plastid-localized multifunctional ACCase is believed to be responsible for fatty acid biosynthesis.

Measurements of the levels of acetyl-ACP and malonyl-ACP in spinach leaves (Post-Beittenmiller et al., 1991) and in isolated spinach and pea chloroplasts (Post-Beittenmiller et al, 1991) in the light and dark have provided evidence that ACCase plays a regulatory role in chloroplast fatty acid biosynthesis in vegetative tissues. In monocots, there also is evidence that ACCase activity regulates fatty acid biosynthesis. Several related cyclohexanedione and aryloxyphenoxypropionate graminicides specifically inhibit monocot multifunctional, plastid-localized ACCase. Complete herbicide inhibition of ACCase results in plant cell death due to cessation of fatty acid biosynthesis (Burton et al., 1989). Based on the apparent flux control coefficient for ACCase in barley and maize leaves exposed to low levels of graminicides, ACCase was shown to be the major flux-controlling enzyme for light-stimulated lipid synthesis in these leaves (Page et al., 1994). Parker et al. (1990b) isolated maize tissue cultures that were selected for growth in the presence of normally lethal concentrations of ACCase inhibiting herbicides. Incubation of wild-type cell cultures in the presence of herbicide inhibited incorporation of acetate into the lipid fraction, while variant cell lines exhibiting up to 20-fold increases in wild-type ACCase activity synthesized fatty acid in the presence of herbicide (Parker et al., 1990b). These results provide further evidence that ACCase is rate-limiting in monocot vegetative tissues.

Monocot plastid-localized, multifunctional ACCase is likely involved in regulating seed oil deposition. Herbicide-resistant maize mutant plants were isolated using tissue culture selection (Parker et al., 1990a). The herbicide-resistant phenotype of these plants is conferred by mutations converting plastid-localized, multifunctional, herbicide-sensitive ACCase to herbicide-resistant ACCase (Marshall et al, 1992; Parker et al., 1990a). Two mutant genes for herbicide-insensitive ACCase, *Acc1* and *Acc2*, have been characterized and both are partially dominant (VanDee et al, 1992). Mutant alleles of *Acc1* were used to study the role of ACCase in regulating kernel development and fatty acid synthesis. ACCase activity in corn embryos increased during development paralleling the reported time course of oil

deposition in this tissue (Somers et al., 1993). Furthermore, the maximum value of corn embryo ACCase was two-fold greater than the average ACCase activity determined in seedling leaf extracts, suggesting that the enzyme was involved in elevated synthesis of fatty acids required for oil deposition in the embryo. Using the herbicide-insensitivity of the mutant ACCase as a marker for the *Acc1* gene, we determined the tissue-specific expression pattern of that gene. ACCase activity assayed in extracts of developing kernels harvested from herbicide-resistant plants was tested for herbicide inhibition. The embryo and endosperm ACCase activities exhibited similar reductions of herbicide inhibition as ACCase extracted from the leaves of these herbicide-resistant mutants, indicating that the *Acc1* gene was the major ACCase expressed in the embryo and endosperm of the developing corn kernel. To further investigate this gene-enzyme relationship, homozygous herbicide-resistant and wild-type susceptible kernels were isolated four days after pollination and cultured *in vitro* to maturity in the presence and absence of a range of herbicide concentrations (Somers et al., 1993). Wild-type kernels cultured on all levels of herbicide tested were shrunken and ceased development presumably because of herbicide inhibition of kernel ACCase activity and thus fatty acid synthesis. Mutant kernels developed normally in the presence of herbicide, indicating that the *Acc1*-encoded ACCase is the major isoform expressed in the developing kernel. The results from the corn studies indicate that multifunctional, plastic-localized ACCase is a rate-limiting enzyme in fatty acid biosynthesis in vegetative and seed tissues.

Complete coding sequences of several multifunctional ACCases from higher plants have been described. They show high amino acid homology within the ATP, carboxybiotin, and acetyl-CoA binding domains and an overall amino acid similarity of 70% or greater. Maize multifunctional ACCase isoforms are encoded by at least four genes, A1, A2, B1, and B2 (Egli et al., 1997). Type A cDNAs are > 98% identical, and are 95% identical to a type B cDNA. Types A and B encode highly similar N-termini and are both likely to encode plastid-targeted isoforms (Egli and Gengenbach, 1996; Egli et al., 1997; Egli et al., 1995). Herbicide resistance in corn (Marshall et al., 1992; VanDee et al., 1992) has been mapped to the same chromosomal locations as an ACCase cDNA probe with high identity to both A and B types (Caffrey et al., 1995; Egli

et al., 1994; VanDee et al., 1992). Herbicide-tolerant ACCase is expressed in developing corn kernels (Somers et al., 1993) and both A- and B-type cDNAs are found in immature embryos at the time of peak ACCase activity (Egli and Gengenbach, 1996; Somers et al., 1993). In hexaploid wheat, six cDNAs encoding cytosolic ACCase were attributed either to three homoeologous sets of duplicate genes or to allelic differences in each genome (Podkowlinski et al., 1996). Hexaploid wheat also contains three transcriptionally active genes encoding plastidic ACCase with ≥ 98% exon sequence identity. These genes are located on homoeologous chromosomes (Gornicki et al., 1997).

Oat is used for both human food and animal feed. Crop improvement goals for oat involve two divergent end uses in that high oil oat is desired as an animal feed whereas a lower oil oat is desired to make low-fat oat products for human consumption. Although progress in decreasing oil content in oat can be achieved by plant breeding, a genetic engineering approach may also be used to create a single, dominant low-oil gene that can be introduced into any oat variety either directly by genetic engineering or via plant breeding for oil reduction in contemporary and future cultivars. Low oil in oat may be a value-added trait for the crop that may allow processors to produce low-fat oat products that contain previously identified cholesterol-lowering constituents including, but not limited to, β-glucans, thus making oats a more healthful human food.

Targeted antisense down-regulation of ACCase in the endosperm using an endosperm-specific promoter may avoid problems that would be caused by inhibition of ACCase in the embryo. Determination of the antisense phenotype by detection of reduced ACCase activity and by quantitation of groat oil content in transgenic lines provides direct evidence for the involvement of ACCase in oat seed oil deposition.

Current oat varieties contain about 7% oil which causes rancidity problems during processing and results in high-fat food products. To reduce oil (triglyceride) content in groats, ACCase cDNA, e.g., oat ACCase DNA, is employed in an antisense orientation to specifically down-regulate oat endosperm ACCase activity and thus reduce groat oil content. Preferably, a plant having a dominantly inherited low-oil gene useful for production of low-oil

varieties is prepared. As oat oil is primarily localized in the starchy endosperm, down-regulation of endosperm ACCase activity is less likely to cause deleterious effects on the embryo. Moreover, converting oil reduction to a single, dominantly inherited antisense trait is useful in rapid production of low oil varieties in this allohexaploid. Seed from transgenic plants are analyzed for expression of the transgene during development and for oil content at maturity. In addition, low oil oat varieties are produced.

Reduction of groat oil content may decrease processing problems associated with oil rancidity and can decrease caloric value resulting in a lower fat food. Reduced-fat oatmeal and other oat products may increase the use of oat as a human food and create a value-enhanced crop for producers of the low-fat oat. In addition, the preparation of high-energy (high oil) oat feed through use of a high-oil gene is also contemplated.

Isolation and Characterization of Oat ACCase cDNAs.

An oat groat cDNA clone was isolated based on its hybridization to a maize cDNA fragment which included portions of the less conserved mid-peptide sequence and the transcarboxylase domain (Shorrosh et al., 1994). Sequence identity indicated that the oat cDNA (3031 bp) corresponded to maize ACCase nt 3124-6169 (Egli et al., 1995). The 1010-aa predicted polypeptide was nearly identical to portions of wheat (89%) and maize (84%) plastidic multifunctional ACCases; it had lower identity to cytosolic ACCases, including wheat (67.5%) and alfalfa (64.6%) (Gornicki et al., 1994; Shorrosh et al., 1994). Therefore, the oat-91 cDNA is likely to encode a portion of a plastid-targeted, multifunctional ACCase polypeptide.

An amplified 'Prairie' oat developing groat cDNA library (R. Skadsen, USDA, Madison, Wisconsin) cloned into the *EcoR*I site of IZAPII (Stratagene) was screened with a [^{32}P-dCTP]-labeled maize ACCase cDNA probe (Ausabel et al., 1997) derived from an *EcoR*I digest of clone 18-5 (nt 2020-5927; Egli et al., 1995). One to three plaques/plate of eleven partially purified positives were eluted in SM medium plus CHC13. *In vivo* excision was carried out to transform SOLR cells with pBlueScript plasmids carrying oat cDNA inserts, according to the manufacturer's instructions (Stratagene Corp.). Five colonies per transformation reaction were rescreened (as colony lifts) with

an AflII-BbrPI fragment of maize ACCase cDNA (4281-5797; Egli et al., 1995). One clone, oat-91, hybridized to the probe. Oat-91 was sequenced using the ABI Prizm system (Perkin-Elmer Corp.) at the Advanced Genetic Analysis Center (University of Minnesota, St. Paul, Minnesota). Sequence identity indicated the 3.05-kb *EcoR*I insert of oat-91 corresponded to maize ACCase nt 3124-6169 (Egli et al., 1995). Oat ACCase cDNA sequence was compared to other plant ACCases with the BESTFIT program (BESTFIT, Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisconsin).

For PCR, Pileup alignments of maize, wheat, barley, and rice ACCase coding sequences (GCG, Madison, Wisconsin; 12, 18, F, R) were used to identify conserved regions (i) for design of new primers or (ii) regions of identity to available maize ACCase oligos, to be used for PCR amplification of oat ACCase. Two maize primers, PCR2 (5′-ggcagagcaa agcttggagg) and PCR1 (5′-tccaccagag aagcctctcc) appeared likely to amplify oat cDNA based on their identity to other monocots and previous use to amplify soybean ACCase. These primers are located within the transcarboxylase domain, surrounding the acetyl-CoA binding site of ACCase (50). An amplified 'Prairie' oat seeding leaf cDNA library cloned into the *EcoR*I site of IZAPII (Skadsen, University of Wisconsin) was used as a source of cDNA. One I of the oat cDNA library was amplified with maize primers (PCR2/PCR1), using the Expand High Fidelity PCR system (BMB) in a GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer) after denaturing phage heads at 94°C for 10 minutes. The single, 222-bp product was purified by Wizard PCR Plus (Promega Corp.) and sequenced in an AB1377 (Perkin-Elmer) using PCR1 as primer. The resulting sequence appeared to be from oat since it had high identity to maize plastidic ACCase (89%; 4, 12, Egli, unpublished). Additional 5′ cDNA was obtained by (i) PCR of the oat cDNA library with T3/PCR1 primers to generate a complex band mixture and (ii) reamplification of 0.1 I products with maize ACCase primers 1324+ (5′-caatcacgtcagactg)/PCR1 to obtain a 1.9-kb product with the same 3′ sequence as the PCR2/PCR1 product.

To test whether the oat ACCase cDNA clone oat-91 encodes a plastidic isoform, 422 nt of 3′ insert sequence was translated and compared to predicted amino acid sequences of maize plastidic ACCases, a putative maize

cytosolic ACCase, and to multifunctional ACCases from wheat, barley, alfalfa, soybean, *Arabidopsis*, *Brassica*, chicken, human, *Ustilago*, yeast and *Cyclotella*. Aligned, degapped peptides were used to construct a phylogram by the UPGMA method based on Kimura protein distances (GCG, Madison, Wisconsin). The oat ACCase sequence was ≥ 93% identical to plastidic maize and wheat ACCase sequences, but only 78-83% identical to cytosolic ACCases from maize, wheat, or dicots. The corresponding nucleotide identities were 88-92% and 73-78%, respectively. In the phylogram, monocot ACCases were separated from dicots and grouped according to their known or expected cellular locations (Figure 25). Both sequence identity and phylogenetic grouping indicate that oat cDNA encodes a plastidic ACCase.

Additional 5′ sequence is obtained by screening the cDNA library with the most 5′ oat probes or by PCR of the library with near 5′ (upstream) oat plus vector-specific oligonucleotide primers. Alternatively, the 5′ end of ACCase message may be obtained by 5′ RACE PCR (Frohman, 1993). Total RNA is isolated from developing groats, and digested with DNAse. Complementary DNA is synthesized with Superscript II reverse transcriptase (Gibco BRL) by priming with oat ACCase primers. PCR amplification of cDNA utilizes oat primers plus 5′ maize primers. Because chloroplast transit peptide sequences are not well conserved between ACCase genes of maize, wheat, and *Brassica*, or between different gene families of a single species, and because 5′ non-coding sequences also are unlikely to be conserved, completion of the 5′ sequence of oat ACCase cDNA is accomplished by 5′ RACE-PCR (GIBCO-BRL; Gornicki et al., 1994). 5′ RACE products and downstream sequences are sequenced to verify that a chloroplast transit peptide coding sequence is present and to identify primers that may be used to generate a complete or partial oat ACCase cDNA product by PCR. Oat cDNA is reamplified using either the Expand High Fidelity or Long Template PCR kits (Boehringer Mannheim Biochemicals), depending on the length of desired product. Products are blunt-cloned into pBluescript (Stratagene) or a similar vector, or extended with dATP and cloned into pGEM-T (Promega). Adjacent sites in vector polycloning regions or unique sites introduced by PCR are utilized to move oat ACCase cDNA into expression vectors.

Co-segregation of Oat ACCase cDNA and a Major Groat Oil QTL

A major quantitative trait locus (QTL) controlling groat oil content in oat was recently identified by the oat genome mapping program at the University of Minnesota (See Example XIV). Single factor ANOVA showed that RFLP marker *Xcdo665B* on linkage group 11 was associated with a QTL accounting for approximately 37% of the variance in groat oil in a Kanota by Ogle mapping population (O'Donoughue et al., 1995). The 1.9 kb oat ACCase cDNA was placed on this map to within 2.6 cM of *Xcdo665B* in the same population (n = 136). This ACCase polymorphism also accounted for about 37% of the variation in groat oil content. These results provide strong support for the hypothesis that ACCase has a major role in determining groat oil content. Either allelic variation in ACCase activity controls oil content in oat groats or a gene(s) closely linked to ACCase is responsible. Suppression of ACCase expression by antisense transformation can provide a direct test of whether ACCase activity indeed has a major effect on groat oil.

Antisense ACCase Transformation Construct Production

Antisense ACCase cDNAs are constructed using standard procedures in the plasmids that are routinely used for oat transformation (Torbert et al., 1995). The cDNA is ligated in antisense orientation relative to an endosperm-specific promoter and is terminated using the nopaline synthase (NOS) 3′ untranslated sequence of the *Agrobacterium tumefaciens* NOS gene, although other termination regions may be employed. This antisense cassette is ligated into a plasmid backbone carrying a neomycin phosphotransferase II (nptII) gene under control of the cauliflower mosaic virus 35S promoter as a selectable marker (Torbert et al., 1995).

An investigation of down-regulated phenotypes conferred by expression of various granule-bound starch synthase antisense constructs in potato indicated that the size of the antisense transcript did not affect efficacy of inhibition (Kuipers et al., 1995). Choice of promoter sequences controlling expression of the antisense construct appeared to have important effects in the degree of antisense inhibition of potato granule-bound starch synthase. Effective antisense RNAs have been reported which (relative to the mRNA) are either full length or fragments designed to block 5′ end capping, intron splicing, or the poly

A tail (Guerineau et al., 1994; Inoye, 1988). Preferably, the longest oat ACCase clones are employed in an anti-sense construct.

Complete inhibition of vegetative- or seed-expressed ACCase is likely to be lethal because the enzyme is required for general fatty acid biosynthesis, as shown by the lethality of herbicide inhibition of ACCase in susceptible monocots. In oat, it is not known whether tissue-specific isozymes or homologs of ACCase exist, or if they do, whether they vary significantly in nucleotide sequence. Therefore, it is important to target antisense down-regulation of ACCase to the developing oat endosperm and that the antisense gene exert a partial down-regulation of endosperm ACCase homologs to avoid deleterious effects of possible endosperm ablation due to complete inhibition of ACCase. Because antisense suppression of heterologous genes or members of a gene family can occur (deLang et al., 1993), and because the partial inhibition of plastidic ACCases but not cytosolic ACCases is desired, partial antisense cDNAs designed from functional domain sequences may be used, or conversely, less-conserved regions between functional domains. Comparison of wheat genes for cytosolic and plastidic ACCases (Gornicki et al., 1997; Gornicki et al., 1994) may aid in the design of antisense cDNAs to specifically target plastidic ACCase.

The wheat Dy10 high molecular weight glutenin promoter (Belchi and Anderson, 1994) confers strong expression of GUS in oat and expression is specifically limited to the endosperm. Because of its tissue specificity and the likelihood that its expression pattern does not directly coincide with the expression of ACCase during oat endosperm development, the Dy10 promoter may be useful in restricting the ACCase antisense transcripts to the oat endosperm resulting in a partial inhibition of ACCase expression and the normal development of endosperm cells.

Oat Transformation

Elite cultivars of oat can be transformed and on average one transgenic tissue culture is produced that regenerates fertile, transgenic plants per two microprojectile bombardments. Generally, from 15 to 30 transgenic plants are produced from the microprojectile bombardments conducted during one experiment. The oat ACCase antisense plasmid is introduced into regenerable

oat tissue cultures of oat genotypes that exhibit high oil content using microprojectile bombardment as described by Torbert et al. (1995). Tissue cultures are selected for resistance to paromomycin conferred by the nptII selectable marker gene. Paromomycin-resistant tissue cultures are regenerated and plants analyzed for the nptII protein using an NPTII ELISA kit (3′ > 5′ Inc., Boulder, CO). Plants that express the nptII protein are grown to maturity.

The progeny from at least 100 independent transgenic events are then evaluated.

Characterization of the ACCase Antisense Phenotype

Homozygous T_2 seed are divided for maintenance, advancement to produce greater quantities of seed for more detailed analyses, and for preliminary analysis. Single oat plants may produce up to 100 seed. Therefore, sufficient seed should be available to determine if a transgene phenotype is detectable. Dried mature seed of antisense homozygotes and nontransgenic sister lines are analyzed for oil content initially using nondestructive Near Infrared Reflectance (NIR) spectroscopy. This method requires only 1 g of dehulled groats and is inexpensive. NIR is employed to rapidly identify transgenic lines that exhibit lowered oil content. Selected lines identified by NIR are reanalyzed using either NMR or the gravimetric procedure (Peterson and Wood, 1997). If differences in oil content are observed, oil composition is analyzed by gas chromatography. If the results of these analyses warrant further investigation of the materials, a replicated greenhouse trial is conducted to confirm the reduced oil phenotype in the transgenic plants. This test is conducted on T_3 seed produced from multiple T_2 plants of each transgenic line. If a low-oil phenotype is observed, these materials are entered into a breeding program to initiate variety development.

Selected low-oil lines are advanced an additional generation. Seed is isolated at different stages of development and stored under conditions to preserve ACCase activity and mRNA for Northern blot analysis. ACCase activity levels and sense and antisense transcript levels are determined to further characterize the antisense phenotype of these plants.

While oat is considered a beneficial alternative crop with multiple uses, production acreage continues to shrink due to poor profitability and

international competition. Yet oat is used as a high quality animal feed and for production of nutritious human food. Numerous positive human health qualities are attributed to consumption of oatmeal. Foremost among these is the cholesterol-lowering property of oats. A major problem confronting oat processors is that current oat varieties have elevated oil content that precludes the production of a low-fat oat product. Thus, in the production of oat products such as oatmeal, the high oil content offsets the benefit of the cholesterol-lowering constituents of the product. Oat oil content is relatively easy to manipulate using conventional plant breeding. However, the trend has been to increase oil content of oats for use as animal feed and only recently has low-oil oat been recognized as important for food products.

Thus, oat varieties with reduced oil content may be prepared using genetic engineering. Moreover, a single dominant gene system can vastly simplify conversion of existing agronomically adapted oat varieties to low oil as well as being useful in the production of new low-oil varieties.

Based on the demand by processors for low-oil oat varieties, it is warranted to consider low oil as a value-added trait for oat. Increasing the value of oat may increase the acreage devoted to growing oats in the U.S. because of possible increased profitability of the crop.

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Youngs, V. L., Peterson, D. M., Brown, C. M. Oats. In: Pomeranz, Y. (ed.) Advances in cereal science and technology. Vol. 5. Am. Assoc. Cereal Chem., St. Paul, Minnesota, pp. 49-105 (1982).

Yuzo, M., Takuji, S. Genbank accession #D24786 (rice) (1993).

The complete disclosure of all patents, patent documents and publications cited herein are incorporated herein by reference as if individually incorporated. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described for variations obvious to one skilled in the art will be included within the invention defined by the claims.

WHAT IS CLAIMED IS:

1. An isolated and purified DNA molecule comprising a DNA segment encoding an oat acetyl CoA carboxylase, or a fragment thereof.

2. The DNA molecule of claim 1 which comprises a DNA segment comprising SEQ ID NO:23.

3. The DNA molecule of claim 1 or 2 further comprising a DNA molecule encoding an amino terminal chloroplast transit peptide, wherein the chloroplast transit peptide is operably linked to the DNA encoding the oat acetyl CoA carboxylase.

4. The DNA molecule of claim 1 which encodes a polypeptide comprising SEQ ID NO:24.

5. A method of imparting cyclohexanedione or aryloxyphenoxypropanoic acid herbicide tolerance to a plant tissue comprising:
   (a) introducing an expression cassette comprising a chimeric DNA molecule encoding an oat acetyl CoA carboxylase comprising SEQ ID NO:24, operably linked to a promoter functional in a plant cell into cells of a susceptible plant so as to yield transformed plant cells;
   (b) regenerating the transformed plant cells to provide a differentiated transformed plant; and
   (c) expressing the chimeric DNA molecule encoding the oat acetyl CoA carboxylase in the cells of the differentiated plant in an amount effective to render the plant tolerant to levels of a cyclohexanedione or an aryloxyphenoxypropanoic acid herbicide which are toxic to a susceptible plant which does not comprise the expression cassette.

6. The method of claim 5 wherein the chimeric DNA molecule encodes an acetyl CoA carboxylase, the activity of which is not inhibited by an amount of cyclohexanedione or aryloxyphenoxypropanoic acid herbicide that inhibits the activity of an acetyl CoA carboxylase which is sensitive to inhibition by a cyclohexanedione or aryloxyphenoxypropanoic acid herbicide.

7. The method of claim 5 wherein the chimeric DNA molecule is expressed in the cells at about a 2′ to about a 20-fold amount over that of a cyclohexanedione or aryloxyphenoxypropanoic acid herbicide-sensitive acetyl CoA carboxylase.

8. The method of claim 5 wherein the chimeric DNA molecule comprises a DNA sequence comprising SEQ ID NO:23.

9. A transformed plant prepared by the method of claim 5.

10. A transformed seed of the transformed plant of claim 9.

11. A transformed monocot plant, which plant is tolerant to herbicides comprising:

(a) an acetyl CoA carboxylase gene that encodes an acetyl CoA carboxylase the activity of which is inhibited by a cyclohexanedione or aryloxyphenoxypropanoic acid herbicide, and

(b) a recombinant DNA molecule encoding an oat acetyl CoA carboxylase operably linked to a promoter functional in a plant, wherein the recombinant DNA molecule is expressed in the plant in an amount effective to confer tolerance to the levels of a cyclohexanedione herbicide or an aryloxyphenoxypropanoic acid herbicide which inhibit the activity of the acetyl CoA carboxylase of step (a).

12. A method for altering the oil content in a plant comprising:

(a) introducing an expression cassette comprising a recombinant DNA molecule encoding an oat acetyl CoA carboxylase operably linked to a promoter functional in a plant cell into the cells of a plant so as to yield transformed plant cells; and

(b) regenerating the transformed plant cells to provide a differentiated transformed plant, wherein the plant expresses the DNA molecule encoding the oat acetyl CoA carboxylase in cells of said plant so as to alter the oil content of the differentiated transformed plant cells relative to the oil content of cells of a corresponding untransformed plant.

13. The method according to claim 12, wherein the recombinant DNA molecule is expressed at about a 2- to about a 20-fold increase over the level of the native acetyl CoA carboxylase.

14. The method according to claim 13 wherein the oil content of the plant cells is increased about 1.2- to about 20-fold.

15. A transformed plant having an altered oil content in the plant cells comprising: a recombinant DNA molecule encoding an oat acetyl CoA carboxylase, wherein the recombinant DNA molecule is expressed in the cells of the plant in an amount of acetyl CoA carboxylase effective to alter the oil content of the plant cells relative to the oil content in the cells of a corresponding untransformed plant.

16. The transformed plant of claim 15 wherein the transformed plant has an increase in oil content of about 1.2- to about 20-fold in its leaves, seeds, or fruit above that present in a corresponding untransformed plant.

17. A transformed plant prepared by the method of claim 12.

18. A seed of the transformed plant of claim 17.

19. The seed of claim 18 wherein the oil content is increased in the endosperm.

20. A method of introducing an exogenous oat acetyl CoA carboxylase gene into a host cell comprising:

(a) introducing to host cells *in vitro* an expression cassette comprising a DNA molecule encoding an oat acetyl CoA carboxylase operably linked to a promoter functional in the host cell so as to yield transformed cells; and

(b) identifying transformed host cells which express the DNA molecule.

21. The method of claim 20 wherein the host cells are plant cells.

22. The method of claim 21 wherein the plant cells can be regenerated into a differentiated plant.

23. The method of claim 21 wherein the plant cells are monocot cells.

24. The method of claim 21 wherein the plant cells are dicot cells.

25. The method of claim 12, wherein the DNA molecule encoding the oat acetyl CoA carboxylase is introduced into plant cells by a method selected from the group consisting of electroporation, microinjection, protoplast transformation, microprojectile bombardment, and liposomal encapsulation, *Agrobacterium*-mediated transformation.

26. A method for altering the oil content in a plant comprising:

(a) introducing an expression cassette comprising a recombinant DNA molecule encoding an antisense oat acetyl CoA carboxylase transcript operably linked to a promoter functional in a plant cell into the cells of a plant so as to yield transformed plant cells; and

(b) regenerating the transformed plant cells to provide a differentiated transformed plant, wherein the transcript is expressed in the cells of the differentiated transformed plant so as to alter the oil content of the plant cells relative to the cells of a corresponding untransformed plant.

27. A transformed plant having an altered oil content in the plant cells comprising: a recombinant DNA molecule encoding an antisense oat acetyl CoA carboxylase transcript wherein the transcript is expressed in the cells of the plant so as to alter the oil content of the plant cells relative to the cells of a corresponding untransformed plant.

28. The method of claim 12 or 26 wherein the recombinant DNA molecule further comprises a DNA segment encoding a chloroplast transit peptide which is operably linked to the DNA molecule encoding the acetyl CoA carboxylase.

29. The transformed plant of claim 15 or 27 wherein the recombinant DNA molecule further comprises a DNA segment encoding a chloroplast transit peptide which is operably linked to the DNA molecule encoding the acetyl CoA carboxylase.

30. The method of claim 12 or 26 wherein the recombinant DNA molecule further comprises a DNA segment comprising a endosperm-specific promoter.

31. The plant of claim 15 or 27 wherein the recombinant DNA molecule further comprises a DNA segment comprising a endosperm-specific promoter.

32. The method of claim 12 or 26 wherein the cells are monocot plant cells.

33. The method of claim 32 wherein the cells are wheat, rice, maize, barley, millet, sorghum, sugarcane, rye, oat or timothy cells.

34. The method of claim 12 or 26 wherein the cells are dicot plant cells.

35. The method of claim 34 wherein the cells are soybean, rape, sunflower, tobacco, Arabidopsis, petunia, pea, Canola, bean, tomato, bean, potato, lettuce, spinach, alfalfa, cotton or carrot.

36. The method of claim 12, 20, or 25 wherein the DNA molecule encodes a polypeptide comprising SEQ ID NO:24.

37. The plant of claim 11, 15, or 27 wherein the DNA molecule encodes a polypeptide comprising SEQ ID NO:24.

38. An expression cassette comprising an endosperm-specific transcriptional control region operably linked to a DNA encoding a plant ACCase, or a biologically active subunit thereof.

39. The expression cassette of claim 38 wherein the endosperm-specific transcriptional control region comprises Dy10.

40. The plant of claim 15 which has increased lipid content, lipid biosynthesis or lipid deposition in groat.

41. The plant of claim 27 which has decreased lipid content, lipid biosynthesis or lipid deposition in groat.

42. The plant of claim 15 which has increased storage of seed lipid in endosperm.

43. The plant of claim 27 which has decreased storage of seed lipid in endosperm.

44. A method to identify an oat plant having altered groat oil content, comprising:

(a) contacting a probe comprising at least a portion of a nucleic acid sequence encoding oat acetyl CoA carboxylase with a nucleic acid sample from a plant in an amount sufficient to form complexes, wherein the sample comprises genomic nucleic acid; and

(b) detecting or determining the amount of complex formation.

45. A method to identify a plant having altered oil content, β-glucan content, seed weight, starch content or protein content, comprising:

(a) contacting a probe comprising at least a portion of a nucleic acid sequence encoding oat acetyl CoA carboxylase with a sample from a plant in an amount sufficient to form complexes, wherein the sample comprises genomic nucleic acid; and

(b) detecting or determining the amount of complex formation.

46. The method of claim 45 wherein the plant is an oat, maize or wheat plant.

47. A method for marker-assisted selection of plants having desired properties, comprising:

(a) contacting a probe comprising at least a portion of a nucleic acid sequence encoding oat acetyl CoA carboxylase with a sample from a plant in an amount sufficient to form complexes, wherein the sample comprises genomic nucleic acid; and

(b) detecting or determining the amount of complex formation.

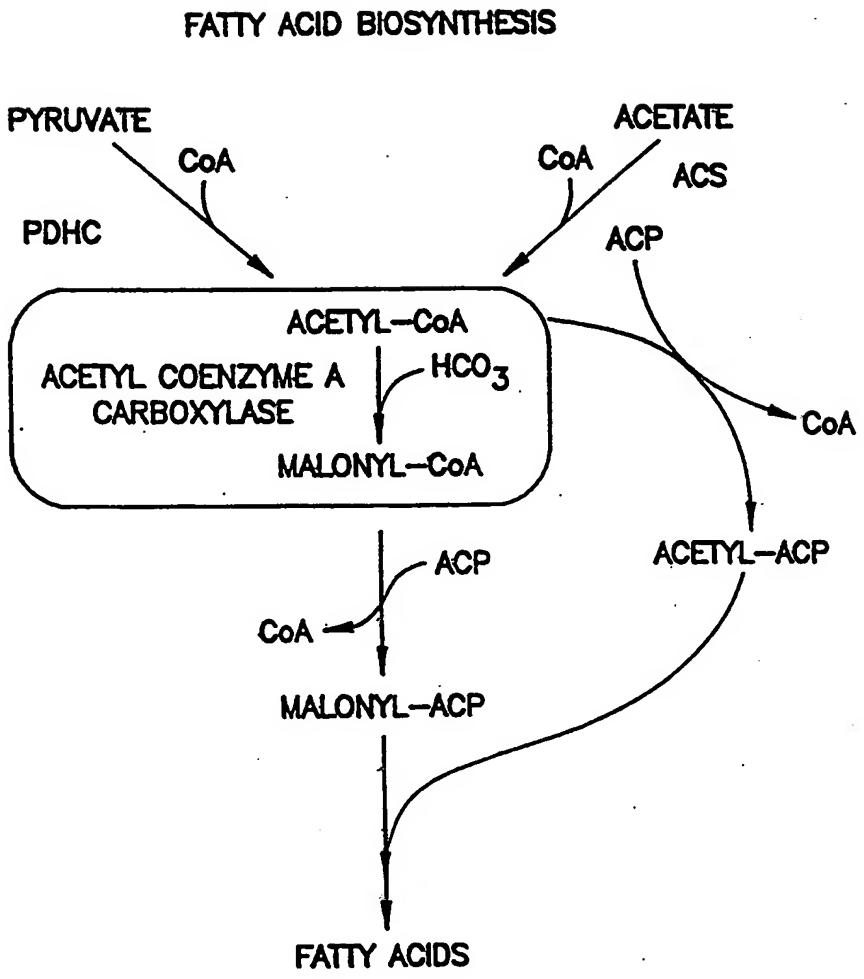
48. An isolated and purified nucleic acid segment associated with altered oil content or composition in monocots.

49. A method to map a restriction fragment length polymorphism in plants, comprising:

(a) contacting a probe comprising at least a portion of a nucleic acid sequence encoding oat acetyl CoA carboxylase with a sample from a plant in an amount sufficient to form complexes, wherein the sample comprises genomic nucleic acid; and

(b) detecting or determining the amount of complex formation.

50. The method of claim 44, 45, 47 or 49 wherein the genomic nucleic acid is from oat, maize, rice or wheat.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PYRUVATE
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PDHC
ACETATE
CoA
ACS
ACP
ACETYL–CoA
ACETYL COENZYME A CARBOXYLASE
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MALONYL–CoA
CoA
ACP
CoA
ACETYL–ACP
MALONYL–ACP
FATTY ACIDS

FIG. 1

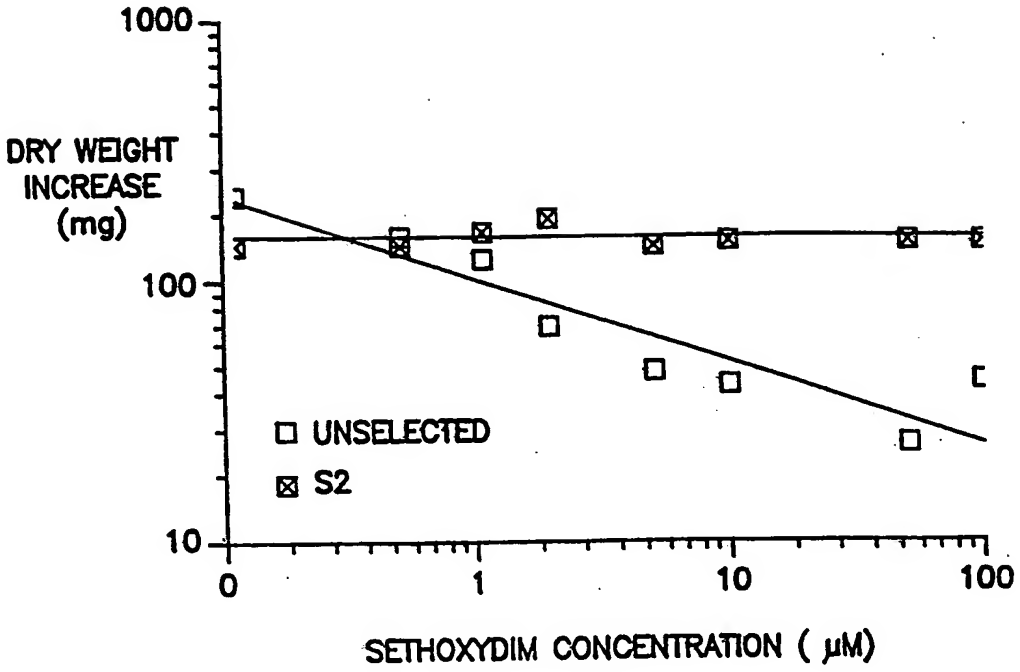
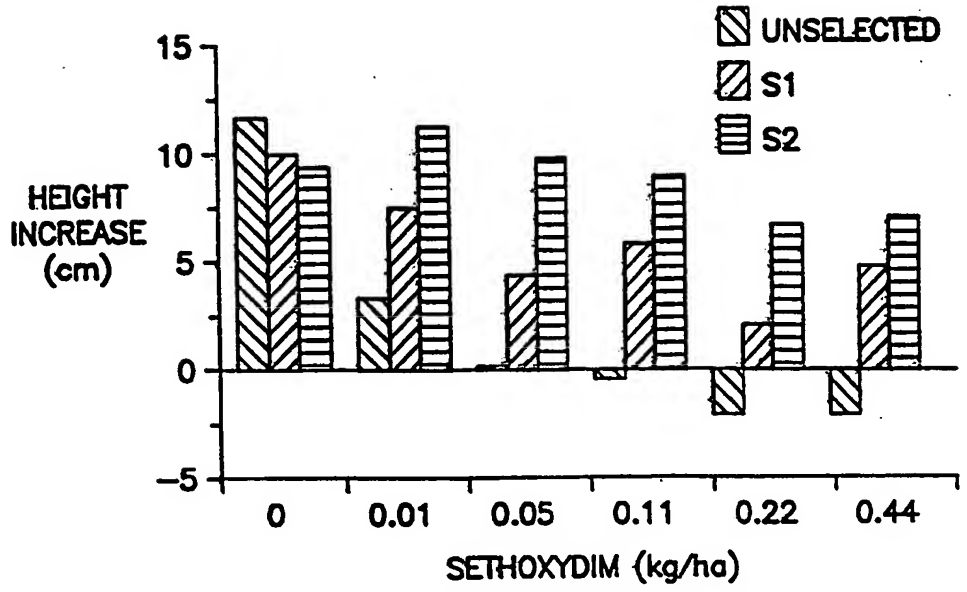

1000
100
10
DRY WEIGHT INCREASE (mg)
UNSELECTED
S2
0
1
10
100
SETHOXYDIM CONCENTRATION (µM)

FIG. 2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S1
S2
15
10
5
0
−5
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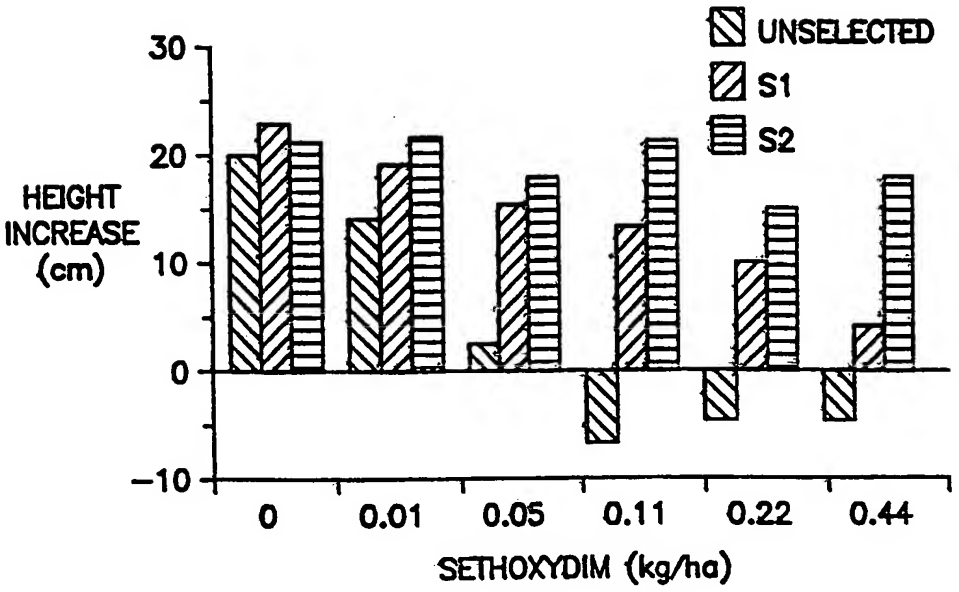
0.01
0.05
0.11
0.22
0.44
SETHOXYDIM (kg/ha)

FIG. 3


UNSELECTED
S1
S2
HEIGHT INCREASE (cm)
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0.05
0.11
0.22
0.44
SETHOXYDIM (kg/ha)

FIG. 4

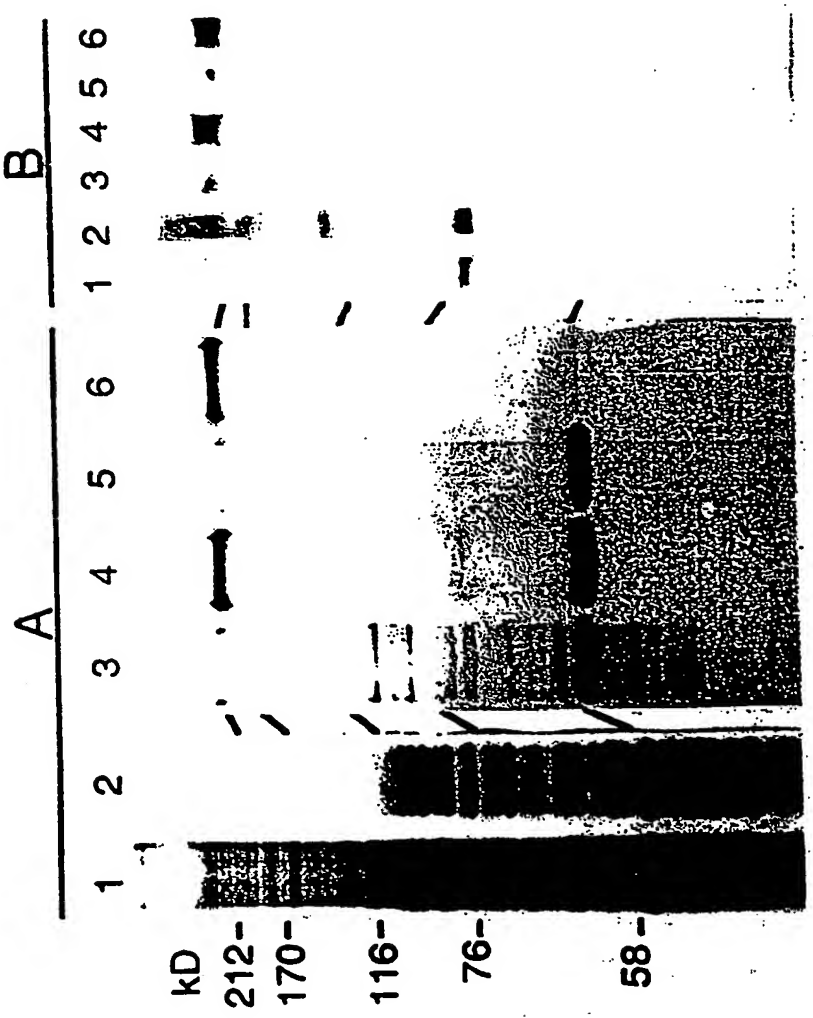


FIG.5

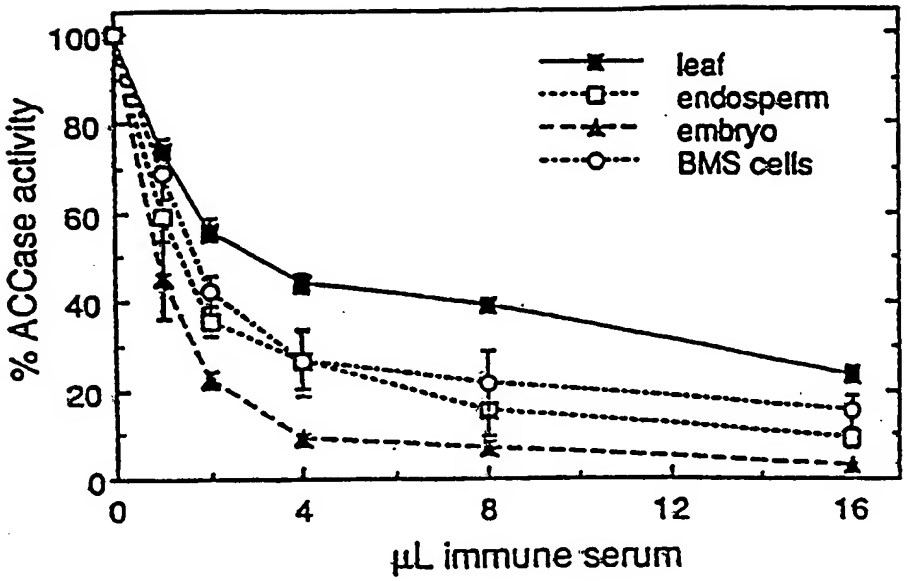

% ACCase activity
100
80
60
40
20
0
0
4
8
12
16
µL immune serum
leaf
endosperm
embryo
BMS cells

FIG. 6

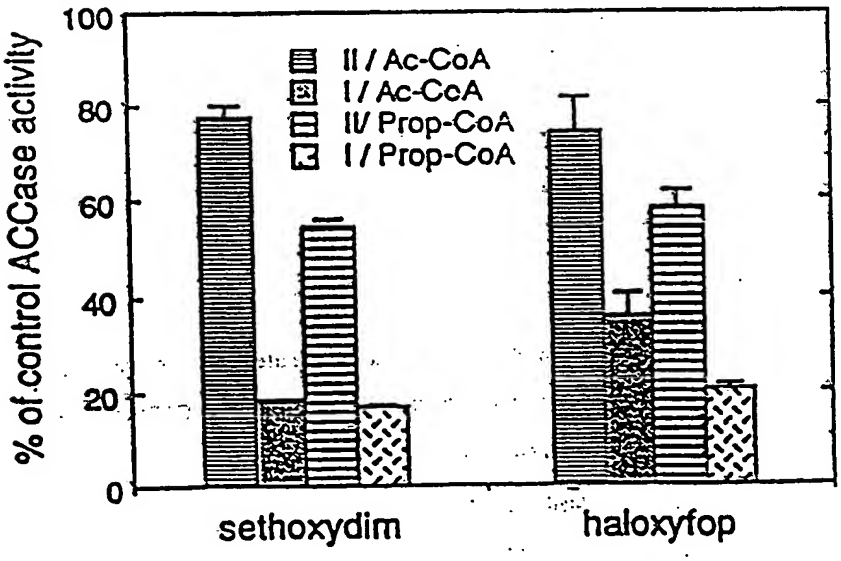
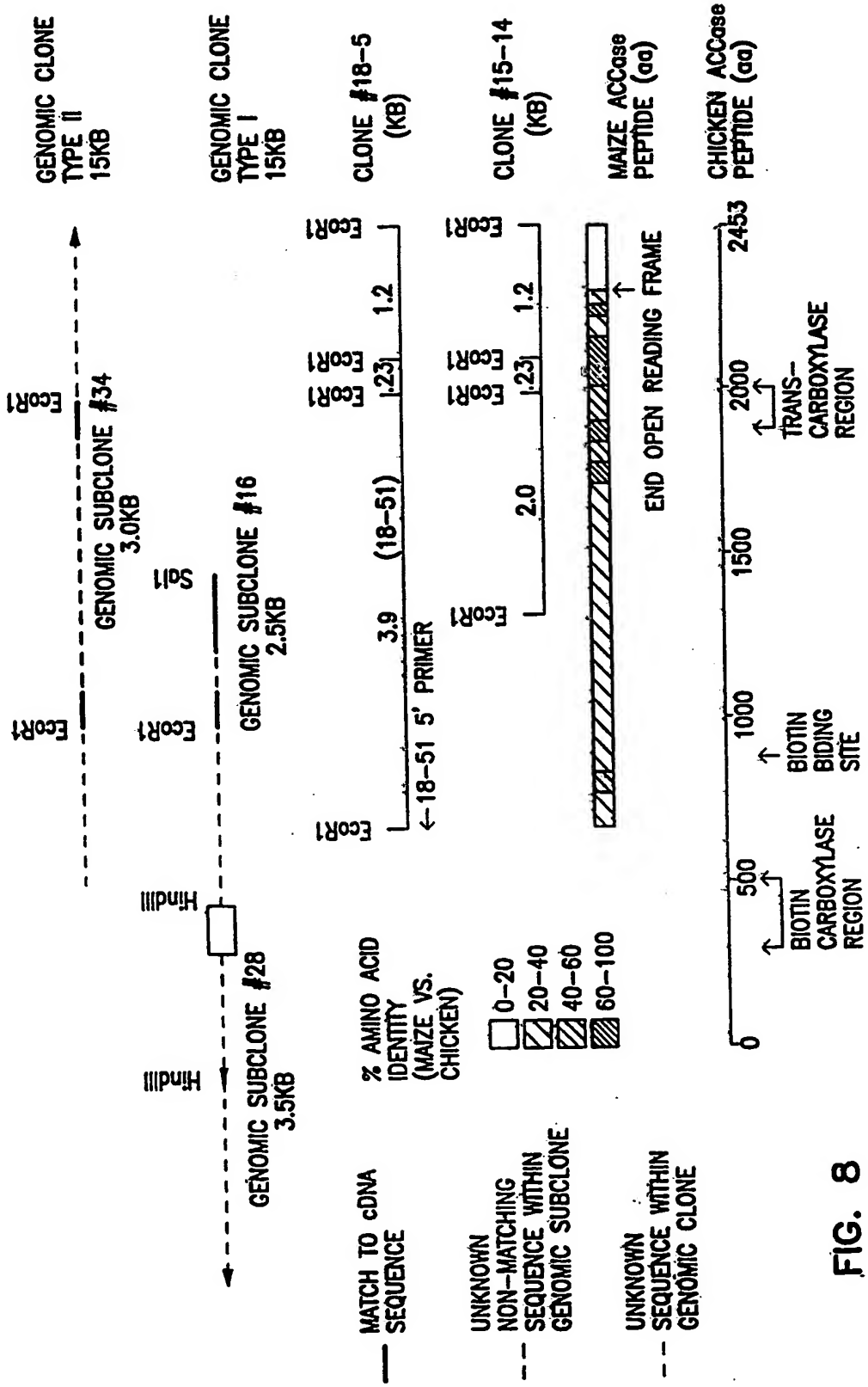

% of control ACCase activity
100
80
60
40
20
0
II / Ac-CoA
I / Ac-CoA
II/ Prop-CoA
I / Prop-CoA
sethoxydim
haloxyfop

FIG. 7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GENOMIC SUBCLONE #16 2.5KB
GENOMIC SUBCLONE #28 3.5KB
EcoR1
Sal1
HindIII
GENOMIC CLONE TYPE I 15KB
CLONE #18-5 (KB)
3.9
(18-51)
.23
1.2
←18-51 5' PRIMER
CLONE #15-14 (KB)
2.0
MAIZE ACCase PEPTIDE (aa)
END OPEN READING FRAME
CHICKEN ACCase PEPTIDE (aa)
0
500
1000
1500
2000
2453
BIOTIN CARBOXYLASE REGION
BIOTIN BIDING SITE
TRANS-CARBOXYLASE REGION
MATCH TO cDNA SEQUENCE
% AMINO ACID IDENTITY (MAIZE VS. CHICKEN)
0-20
20-40
40-60
60-100
UNKNOWN NON-MATCHING SEQUENCE WITHIN GENOMIC SUBCLONE
UNKNOWN SEQUENCE WITHIN GENOMIC CLONE

FIG. 8

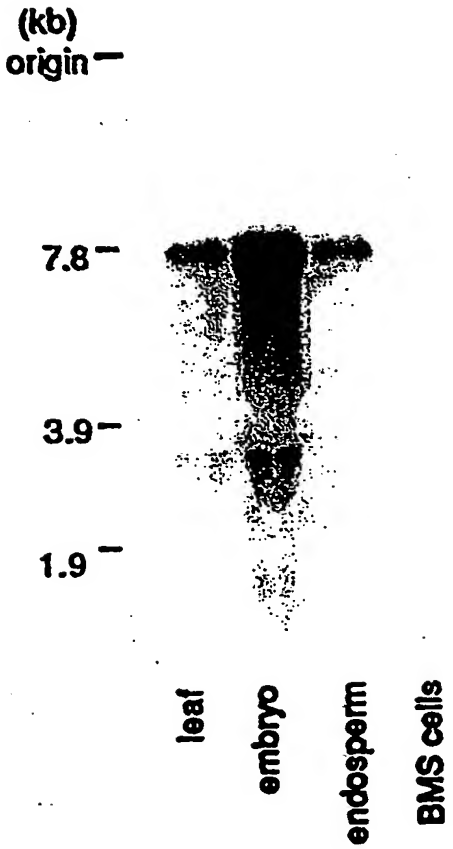

FIG. 9

AGA GAT GAA GCT CGC ATG CCA ATG CGC CAC ACA TTC CTC TGG TTG GAT
GAC AAG AGT TGT TAT GAA GAA GAG CAG ATT CTC CGG CAT GTG GAG GCT
CCC CTC TCT ACA CTT CTT GAA TTG GAT AAG TTG AAG GTG AAA GGA TAC
AAT GAA ATG AAG TAT ACT CCT TCG CGT GAC CGC CAA TGG CAT ATC TAC
ACA CTA AGA AAT ACT GAA AAC CCC AAA ATG TTG CAT AGG GTG TTT TTC
CGA ACT ATT GTC AGG CAA CCC AAT GCA GGC AAC AAG TTT AGA TCG GCT
CAG ATC AGC GAC GCN AAG GTA GGA TGT CCC GAA GAA TCT CTT TCA TTT
ACA TCA AAT AGC ATC TTA AGA TCA TTG ATG ACT GCT ATT GAA GAA TTA
GAG CTT CAT GCA ATT AGG ACA GGT CAT TCT CAC ATG TAT TTG TGC ATA
CTG AAA GAG CAA AAG CTT CTT GAC CTC ATT CCA TTT TCA GGG AGT ACA
ATT GTT GAT GTT GGC CAA GAT GAA GCT ACC GCT TGT TCA CTT TTA AAA
TCA ATG GCT TTG AAG ATA CAT GAG CTT GTT GGT GCA AGG ATG CAT CAT
CTG TCT GTA TGC CAG TGG GAG GTG AAA CTC AAG TTG GAC TGT GAT GGC
CCT GCA AGT GGT ACC TGG AGA GTT GTA ACT ACA AAT GTT ACT GGT CAC
ACC TGC ACC ATT GAT ATA TAC CGA GAA GTG GAG GAA ATA GAA TCA CAG
AAG TTA GTG TAC CAT TCA GCC AGT TCG TCA GCT GGA CCA TTG CAT GGT
GTT GCA CTG AAT AAT CCA TAT CAA CCT TTG AGT GTG ATT GAT CTA AAG
CGC TGC TCT GCT AGG AAC AAC AGA ACA ACA TAT TGC TAT GAT TTT CCG
CTG GCC TTT GAA ACT GCA CTG CAG AAG TCA TGG CAG TCC AAT GGC TCT
ACT GTT TCT GAA GGC AAT GAA AAT AGT AAA TCC TAC GTG AAG GCA ACT
GAG CTA GTG TTT GCT GAA AAA CAT GGG TCC TGG GGC ACT GCT ATA ATT
CCG ATG GAA CGC CCT GCT GGG CTC AAC GAC ATT GGT ATG GTC GCT TGG
ATC ATG GAG ATG TCA ACA CCT GAA TTT CCC AAT GGC AGG CAG ATT ATT
GTT GTA GCA AAT GAT ATC ACT TTC AGA GCT GGA TCA TTT GGC CCA AGG
GAA GAT GCA TTT TTT GAA ACT GTC ACT AAC CTG GCT TGC GAA AGG AAA
CTT CCT CTT ATA TAC TTG GCA GCA AAC TCT GGT GCT AGG ATT GGC ATA
GCT GAT GAA GTA AAA TCT TGC TTC CGT GTT GGA TGG TCT GAC GAA GGC
AGT CCT GAA CGA GGG TTT CAG TAC ATC TAT CTG ACT GAA GAA GAC TAT
GCT CGC ATT AGC TCT TCT GTT ATA GCA CAT AAG CTG GAG CTA GAT AGT
GGT GAA ATT AGG TGG ATT ATT GAC TCT GTT GTG GGC AAG GAG GAT GGG
CTT GGT GTC GAG AAC ATA CAT GGA AGT GCT GCT ATT GCC AGT GCT TAT
TCT AGG GCA TAT GAG GAG ACA TTT ACA CTT ACA TTT GTG ACT GGG CGG
ACT GTA GGA ATA GGA GCT TAT CTT GCT CGA CTT GGT ATA CGG TGC ATA
CAG CGT CTT GAC CAG CCT ATT ATT TTA ACA GGG TTT TCT GCC CTG AAC
AAG CTC CTT GGG CGG GAA GTG TAC AGC TCC CAC ATG CAG CTT GGT GGT
CCT AAG ATC ATG GCG ACC AAT GGT GTT GTC CAC CTC ACT GTT CCA GAT

FIG. 10

GTC CTT GAA GGT GTT TCC AAT ATA TTG AGG TGG CTC AGC TAT GTT CCT
GCA AAC ATT GGT GGA CCT CTT CCT ATT ACC AAA CCT CTG GAC CCT CCA
GAC AGA CCT GTT GCT TAC ATC CCT GAG AAC ACA TGC GAT CCA CGT GCA
GCT ATC TGT GGT GTA GAT GAC AGC CAA GGG AAA TGG TTG GGT GGT ATG
TTT GAC AAA GAC AGC TTT GTG GAG ACA TTT GAA GGA TGG GCA AAA ACA
GTG GTT ACT GGC AGA GCA AAG CTT GGA GGA ATT

FIG. 10 (continued)

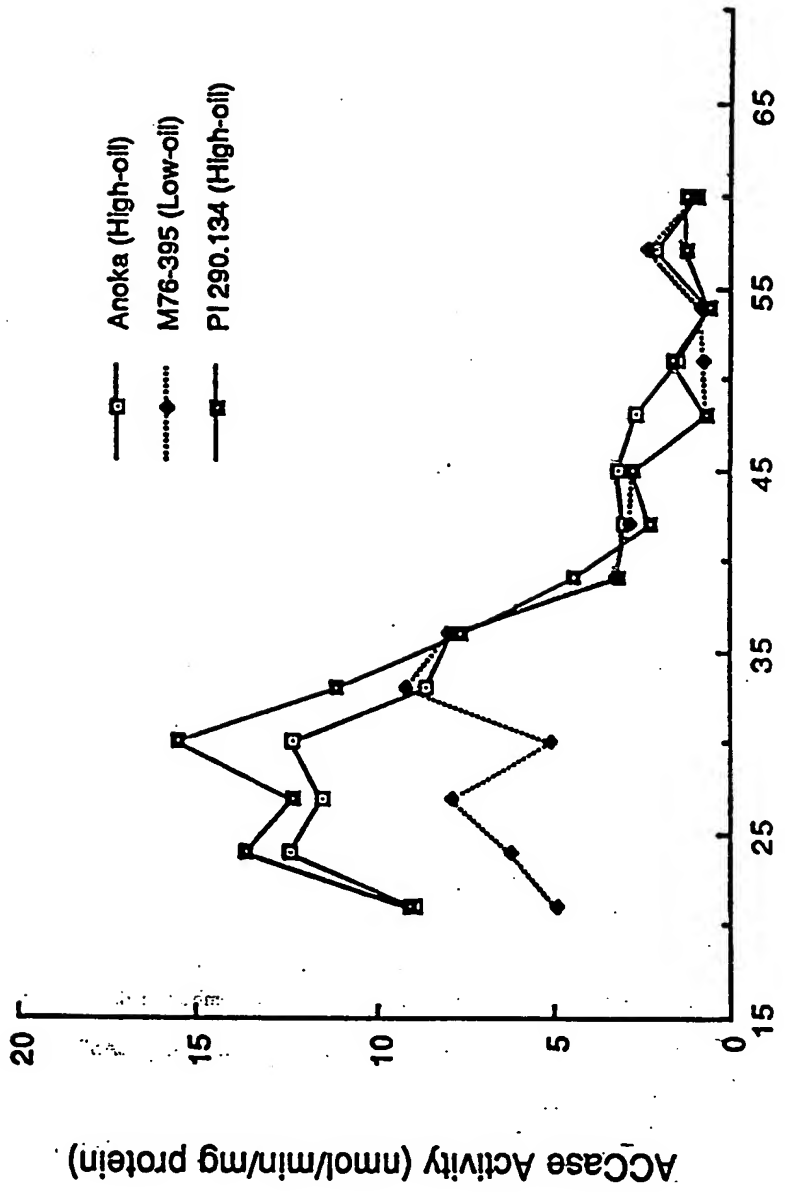
Anoka (High-oil)
M76-395 (Low-oil)
PI 290.134 (High-oil)
ACCase Activity (nmol/min/mg protein)
0
5
10
15
20
15
25
35
45
55
65
Days After Flower

FIG. 11

ACCase A cloning strategy and position of start and stop codons

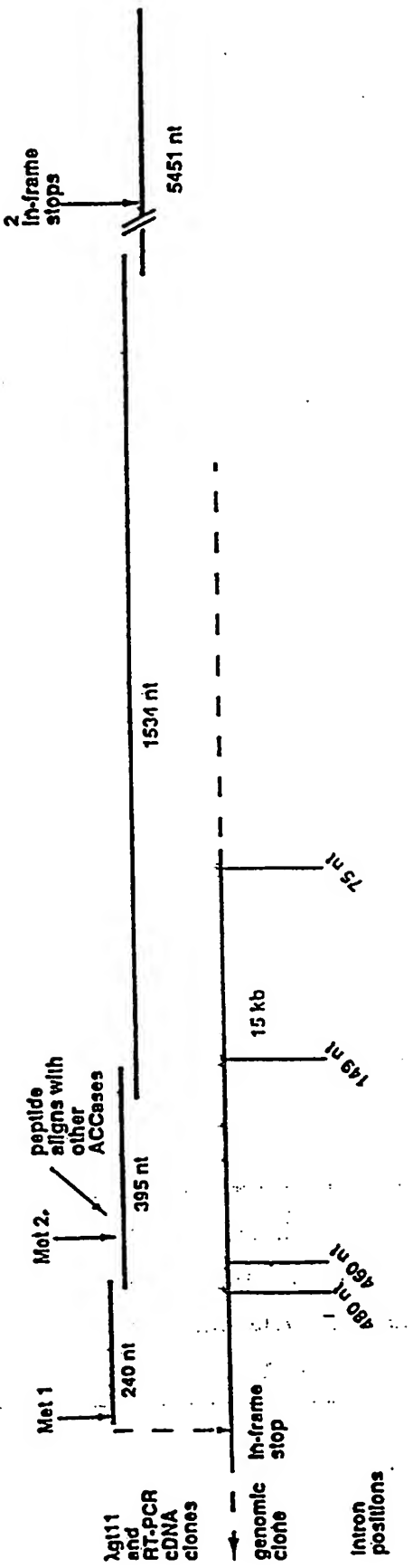

FIG. 12

```
   1  GGTCTTCAAT TGTGCTGTCT GGGCCACGGA ACGACAATGT CACAGCTTGG
  51  ATTAGCCGCA GCTGCCTCAA AGGCCTTGCC ACTACTCCCT AATCGCCAGA
 101  GAAGTTCAGC TGGGACTACA TTCTCATCAT CTTCATTATC GAGGCCCTTA
 151  AACAGAAGGA AAAGCCATAC TCGTTCACTC CGTGATGGCG GAGATGGGGT
 201  ATCAGATGCC AAAAAGCACA GCCAGTCTGT TCGTCAAGGT CTTGCTGGCA
 251  TTATCGACCT CCCAAGTGAG GCACCTTCCG AAGTGGATAT TTCACATGGA
 301  TCTGAGGATC CTAGGGGGCC AACAGATTCT TATCAAATGA ATGGGATTAT
 351  CAATGAAACA CATAATGGAA GACATGCCTC AGTGTCCAAG GTTGTTGAAT
 401  TTTGTGCGGC ACTAGGTGGC AAAACACCAA TTCACAGTAT ATTAGTGGCC
 451  AACAATGGAA TGGCAGCAGC AAAATTTATG AGGAGTGTCC GGACATGGGC
 501  TAATGATACT TTTGGATCTG AGAAGGCAAT TCAACTCATA GCTATGGCAA
 551  CTCCGGAAGA CATGAGGATA AATGCAGAAC ACATTAGAAT TGCTGACCAA
 601  TTCGTAGAGG TGCCTGGTGG AACAAACAAT AATAACTACG CCAATGTTCA
 651  ACTCATAGTG GGGATGGCAC AAAAACTAGG TGTTTCTGCT GTTTGGCCTG
 701  GTTGGGGTCA TGCTTCTGAG AATCCTGAAC TGCCAGATGC ATTGAGCGCA
 751  AAAGGGATCG TTTTTCTTGG CCCACCTGCA TCATCAATGA ATGCTTTGGG
 801  AGATAAGGTC GGCTCAGCTC TCATTGCTCA AGCAGCCGGG GTCCCAACTC
 851  TTGCTTGGAG TGGATCACAT GTTGAAGTTC CATTAGAGTG CTGCTTAGAC
 901  GCGATACCTG AGGAGATGTA TAGAAAAGCT TGCGTTACTA CCACAGAGGA
 951  AGCAGTTGCA AGTTGTCAAG TGGTTGGTTA TCCTGCCATG ATTAAGGCAT
1001  CCTGGGGAGG TGGTGGTAAA GGAATAAGAA AGGTTCATAA TGATGATGAG
1051  GTTAGAGCGC TGTTTAAGCA AGTACAAGGT GAAGTCCCTG GCTGCCCAAT
1101  ATTTGTCATG AGGCTTGCAT CCCAGAGTCG GCATCTTGAA GTTCAGTTGC
1151  TTTGTGATCA ATATGGTAAT GTAGCAGCAC TTCACAGTCG TGATTGCAGT
1201  GTGCAACGGC GACACCAGAA GATTATTGAA GAAGGTCCAG TTACTGTTGC
1251  TCCTCGTGAG ACAGTTAAAG CACTTGAGCA GGCAGCAAGG AGGCTTGCTA
1301  AGGCTGTGGG TTATGTTGGT GCTGCTACTG TTGAGTATCT TTACAGCATG
1351  GAAACTGGAG ACTACTATTT TCTGGAACTT AATCCCCGAC TACAGGTTGA
1401  GCATCCAGTC ACTGAGTGGA TAGCTGAAGT GAATCTGCCT GCAGCTCAAG
1451  TTGCTGTTGG AATGGGCATA CCTCTTTGGC AGATTCCAGA AATCAGACGT
1501  TTCTATGGAA TGGACTATGG AGGAGGGTAT GACATTTGGA GGAAAACAGC
1551  AGCTCTTGCT ACACCATTTA ATTTTGATGA AGTAGATTCT CAATGGCCAA
1601  AGGGCCATTG TGTAGCAGTT AGAATTACTA GTGAGGACCC AGATGATGGT
1651  TTCAAACCTA CTGGTGGGAA AGTGAAGGAG ATAAGTTTTA AAAGCAAGCC
1701  TAATGTTTGG GCCTACTTCT CAGTAAAGTC TGGTGGAGGC ATTCATGAAT
1751  TTGCTGATTC TCAGTTTGGA CATGCTTTTG CATATGGACT CTCTAGACCA
```

FIG. 13

```
1801  GCAGCTATAA CAAACATGTC TCTTGCATTA AAAGAGATTC AGATTCGTGG
1851  AGAAATTCAT TCAAATGTTG ATTACACAGT TGACCTCTTA AAGGCTTCAG
1901  ACTTCAGAGA AAACAAGATC CACACTGGTT GGCTGGATAC AAGAATAGCT
1951  ATGCGTGTTC AAGCTGAGAG GCCCCCATGG TATATCTCAG TGGTTGGAGG
2001  TGCTTTATAT AAAACAGTAA CCACCAATGC AGCCACTGTT TCTGAATATG
2051  TTAGTTATCT CACCAAGGGC CATATTCCAC CAAAGCATAT ATCCCTTGTC
2101  AATTCTACAG TTAATTTGAA TATAGAAGGG AGCAAATACA CAATTGAAAC
2151  TGTAAGGACT GGACATGGTA GCTACAGGTT GAGAATGAAT GATTCAACAG
2201  TTGAAGCGAA TGTACAATCT TTATGTGATG GTGGCCTCTT AATGCAGTTG
2251  GATGGAAACA GCCATGTAAT TTATGCAGAA GAAGAAGCTG GTGGTACACG
2301  GCTTCAGATT GATGGAAAGA CATGTTTATT GCAGAATGAC CATGATCCAT
2351  CGAAGTTATT AGCTGAGACA CCCTGCAAAC TTCTTGGTTT CTTGGTTGCT
2401  GATGGTGCTC ATGTTGATGC GGATGTACCA TACGCGGAAG TTGAGGTTAT
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2501  TGATGTCTGA GGGCCAGGCA TTGCAGGCTG GTGATCTTAT AGCAAGGTTG
2551  GATCTTGATG ACCCTTCTGC TGTGAAAAGA GCTGAGCCAT TTGATGGAAT
2601  ATTTCCACAA ATGGAGCTCC CTGTTGCTGT CTCTAGTCAA GTACACAAAA
2651  GATATGCTGC AAGTTTGAAT GCTGCTCGAA TGGTCCTTGC AGGATATGAG
2701  CACAATATTA ATGAAGTCGT TCAAGATTTG GTATGCTGCC TGGACAACCC
2751  TGAGCTTCCT TTCCTACAGT GGGATGAACT TATGTCTGTT CTAGCAACGA
2801  GGCTTCCAAG AAATCTCAAG AGTGAGTTAG AGGATAAATA CAAGGAATAC
2851  AAGTTGAATT TTTACCATGG AAAAAACGAG GACTTTCCAT CCAAGTTGCT
2901  AAGAGACATC ATTGAGGAAA ATCTTTCTTA TGGTTCAGAG AAGGAAAAGG
2951  CTACAAATGA GAGGCTTGTT GAGCCTCTTA TGAACCTACT GAAGTCATAT
3001  GAGGGTGGGA GAGAGAGCCA TGCACATTTT GTTGTCAAGT CTCTTTTCGA
3051  GGAGTATCTT ACAGTGGAAG AACTTTTTAG TGATGGCATT CAGTCTGACG
3101  TGATTGAAAC ATTGCGGCAT CAGCACAGTA AAGACCTGCA GAAGGTTGTA
3151  GACATTGTGT TGTCTCACCA GGGTGTGAGG AACAAAGCTA AGCTTGTAAC
3201  GGCACTTATG GAAAAGCTGG TTTATCCAAA TCCTGGTGGT TACAGGGATC
3251  TGTTAGTTCG CTTTTCTTCC CTCAATCATA AAAGATATTA TAAGTTGGCC
3301  CTTAAAGCAA GTGAACTTCT TGAACAAACC AAACTAAGTG AACTCCGTGC
3351  AAGCGTTGCA AGAAGCCTTT CGGATCTGGG GATGCATAAG GGAGAAATGA
3401  GTATTAAGGA TAACATGGAA GATTTAGTCT CTGCCCCATT ACCTGTTGAA
3451  GATGCTCTGA TTTCTTTGTT TGATTACAGT GATCGAACTG TTCAGCAGAA
3501  AGTGATTGAG ACATACATAT CACGATTGTA CCAGCCTCAT CTTGTAAAGG
3551  ATAGCATCCA AATGAAATTC AAGGAATCTG GTGCTATTAC TTTTTGGGAA
```

FIG. 13 (continued)

```
3601  TTTTATGAAG GGCATGTTGA TACTAGAAAT GGACATGGGG CTATTATTGG
3651  TGGGAAGCGA TGGGGTGCCA TGGTCGTTCT CAAATCACTT GAATCTGCGT
3701  CAACAGCCAT TGTGGCTGCA TTAAAGGATT CGGCACAGTT CAACAGCTCT
3751  GAGGGCAACA TGATGCACAT TGCATTATTG AGTGCTGAAA ATGAAAGTAA
3801  TATAAGTGGA ATAAGCAGTG ATGATCAAGC TCAACATAAG ATGGAAAAGC
3851  TTAGCAAGAT ACTGAAGGAT ACTAGCGTTG CAAGTGATCT CCAAGCTGCT
3901  GGTTTGAAGG TTATAAGTTG CATTGTTCAA AGAGATGAAG CTCGCATGCC
3951  AATGCGCCAC ACATTCCTCT GGTTGGATGA CAAGAGTTGT TATGAAGAAG
4001  AGCAGATTCT CCGGCATGTG GAGCCTCCCC TCTCTACACT TCTTGAATTG
4051  GATAAGTTGA AGGTGAAAGG ATACAATGAA ATGAAGTATA CTCCTTCGCG
4101  TGACCGCCAA TGGCATATCT ACACACTAAG AAATACTGAA AACCCCAAAA
4151  TGTTGCATAG GGTGTTTTTC CGAACTATTG TCAGGCAACC CAATGCAGGC
4201  AACAAGTTTA GATCGGCTCA GATCAGCGAC GCTGAGGTAG GATGTCCCGA
4251  AGAATCTCTT TCATTTACAT CAAATAGCAT CTTAAGATCA TTGATGACTG
4301  CTATTGAAGA ATTAGAGCTT CATGCAATTA GGACAGGTCA TTCTCACATG
4351  TATTTGTGCA TACTGAAAGA GCAAAAGCTT CTTGACCTCA TTCCATTTTC
4401  AGGGAGTACA ATTGTTGATG TTGGCCAAGA TGAAGCTACC GCTTGTTCAC
4451  TTTTAAAATC AATGGCTTTG AAGATACATG AGCTTGTTGG TGCAAGGATG
4501  CATCATCTGT CTGTATGCCA GTGGGAGGTG AAACTCAAGT TGGACTGTGA
4551  TGGCCCTGCA AGTGGTACCT GGAGAGTTGT AACTACAAAT GTTACTGGTC
4601  ACACCTGCAC CATTGATATA TACCGAGAAG TGGAGGAAAT AGAATCACAG
4651  AAGTTAGTGT ACCATTCAGC CAGTTCGTCA GCTGGACCAT TGCATGGTGT
4701  TGCACTGAAT AATCCATATC AACCTTTGAG TGTGATTGAT CTAAAGCGCT
4751  GCTCTGCTAG GAACAACAGA ACAACATATT GCTATGATTT TCCGCTGGCC
4801  TTTGAAACTG CACTGCAGAA GTCATGGCAG TCCAATGGCT CTACTGTTTC
4851  TGAAGGCAAT GAAAATAGTA AATCCTACGT GAAGGCAACT GAGCTAGTGT
4901  TTGCTGAAAA ACATGGGTCC TGGGGCACTC CTATAATTCC GATGGAACGC
4951  CCTGCTGGGC TCAACGACAT TGGTATGGTC GCTTGGATCA TGGAGATGTC
5001  AACACCTGAA TTTCCCAATG GCAGGCAGAT TATTGTTGTA GCAAATGATA
5051  TCACTTTCAG AGCTGGATCA TTTGGCCCAA GGGAAGATGC ATTTTTTGAA
5101  ACTGTCACTA ACCTGGCTTG CGAAAGGAAA CTTCCTCTTA TATACTTGGC
5151  AGCAAACTCT GGTGCTAGGA TTGGCATAGC TGATGAAGTA AAATCTTGCT
5201  TCCGTGTTGG ATGGTCTGAC GAAGGCAGTC CTGAAGGAGG GTTTCAGTAC
5251  ATCTATCTGA CTGAAGAAGA CTATGCTCGC ATTAGCTCTT CTGTTATAGC
5301  ACATAAGCTG GAGCTAGATA GTGGTGAAAT TAGGTGGATT ATTGACTCTG
5351  TTGTGGGCAA GGAGGATGGG CTTGGTGTCG AGAACATACA TGGAAGTGCT
```

FIG. 13 (continued)

```
5401  GCTATTGCCA GTGCTTATTC TAGGGCATAT GAGGAGACAT TTACACTTAC
5451  ATTTGTGACT GGGCGGACTG TAGGAATAGG AGCTTATCTT GCTCGACTTG
5501  GTATACGGTG CATACAGCGT CTTGACCAGC CTATTATTTT AACAGGGTTT
5551  TCTGCCCTGA ACAAGCTCCT TGGGCGGGAA GTGTACAGCT CCCACATGCA
5601  GCTTGGTGGT CCTAAGATCA TGGCGACCAA TGGTGTTGTC CACCTCACTG
5651  TTCCAGATGT CCTTGAAGGT GTTTCCAATA TATTGAGGTG GCTCAGCTAT
5701  GTTCCTGCAA ACATTGGTGG ACCTCTTCCT ATTACCAAAC CTCTGGACCC
5751  TCCAGACAGA CCTGTTGCTT ACATCCCTGA GAACACATGC GATCCACGTG
5801  CAGCTATCTG TGGTGTAGAT GACAGCCAAG GGAAATGGTT GGGTGGTATG
5851  TTTGACAAAG ACAGCTTTGT GGAGACATTT GAAGGATGGG CAAAAACAGT
5901  GGTTACTGGC AGAGCAAAGC TTGGAGGAAT TCCTGTGGGC GTCATAGCTG
5951  TGGAGACACA GACCATGATG CAGATCATCC CTGCTGATCC AGGTCAGCTT
6001  GATTCCCATG AGCGATCTGT CCCTCGTGCT GGACAAGTGT GGTTCCCAGA
6051  TTCTGCAACC AAGACCGCTC AGGCATTATT AGACTTCAAC CGTGAAGGAT
6101  TGCCTCTGTT CATCCTGGCT AATTGGAGAG GCTTCTCTGG TGGACAAAGA
6151  GATCTCTTTG AAGGAATTCT TCAGGCTGGG TCAACAATTG TCGAGAAGCT
6201  TAGGACATAT AATCAGCCTG CTTTTGTGTA CATTCCTATG GCTGGAGAGC
6251  TTCGTGGAGG AGCTTGGGTT GTGGTCGATA GCAAAATAAA TCCAGACCGC
6301  ATTGAGTGTT ATGCTGAAAG GACTGCCAAA GGTAATGTTC TCGAACCTCA
6351  AGGGTTAATT GAAATCAAGT TCAGGTCAGA GGAACTCCAA GACTGTATGG
6401  GTAGGCTTGA CCCAGAGTTG ATAAATCTGA AAGCAAAACT CCAAGATGTA
6451  AATCATGGAA ATGGAAGTCT ACCAGACATA GAAGGGATTC GGAAGAGTAT
6501  AGAAGCACGT ACGAAACAGT TGCTGCCTTT ATATACCCAG ATTGCAATAC
6551  GGTTTGCTGA ATTGCATGAT ACTTCCCTAA GAATGGCAGC TAAAGGTGTG
6601  ATTAAGAAAG TTGTAGACTG GGAAGAATCA CGCTCGTTCT TCTATAAAAG
6651  GCTACGGAGG AGGATCGCAG AAGATGTTCT TGCAAAAGAA ATAAGGCAGA
6701  TAGTCGGTGA TAAATTTACG CACCAATTAG CAATGGAGCT CATCAAGGAA
6751  TGGTACCTTG CTTCTCAGGC CACAACAGGA AGCACTGGAT GGGATGACGA
6801  TGATGCTTTT GTTGCCTGGA AGGACAGTCC TGAAAACTAC AAGGGGCATA
6851  TCCAAAAGCT TAGGGCTCAA AAAGTGTCTC ATTCGCTCTC TGATCTTGCT
6901  GACTCCAGTT CAGATCTGCA AGCATTCTCG CAGGGTCTTT CTACGCTATT
6951  AGATAAGATG GATCCCTCTC AGAGAGCGAA GTTTGTTCAG GAAGTCAAGA
7001  AGGTCCTTGA TTGATGATAC CAACACATCC AACACAATGT GTGCATGTCA
7051  CATCTTTTTG TTCTAGTACA TACATAGAAG GATATTGCTT GGTCTTGATT
7101  GATCATGTCT GATTTAAGTC GACTATTATT TCTTGGAATT TTCTTTTGGA
7151  CCTGGTGCTA TGGTTGATGG ATGTATATTG GATATGTGCG TTCTGCCAGG
```

FIG. 13 (continued)

```
7201  TGTAAGCACA AAGGTTTAGA CARAMMRARA RCAAGAGCGA GTGAACCTGT
7251  TCTGGTTTTG CAGTGGTTCA GTAAGGCAGA AAGTTGTTAA ACCGTAGTTC
7301  TGAGATGTAT TACCAGTGNC GCCATGCTGT ACTTTTAGGG TGTATAATGC
7351  GGATACAAAT AAACAATTTA GCGGTTCATT AAAGTTTGAA CTCAAATAAC
7401  ATGTTCTTTG TAAGCATATG TACCGTACCT CTACGTGAAA TAAAGTTGTT
7451  GAATTAGCAT TCGAAAAAAA
```

FIG. 13 (continued)

```
   1  MSQLGLAAAA SKALPLLPNR QRSSAGTTFS SSSLSRPLNR RKSHTRSLRD
  51  GGDGVSDAKK HSQSVRQGLA GIIDLPSEAP SEVDISHGSE DPRGPTDSYQ
 101  MNGIINETHN GRHASVSKVV EFCAALGGKT PIHSILVANN GMAAAKFMRS
 151  VRTWANDTFG SEKAIQLIAM ATPEDMRINA EHIRIADQFV EVPGGTNNNN
 201  YANVQLIVGM AQKLGVSAVW PGWGHASENP ELPDALTAKG IVFLGPPASS
 251  MNALGDKVGS ALIAQAAGVP TLAWSGSHVE VPLECCLDAI PEEMYRKACV
 301  TTTEEAVASC QVVGYPAMIK ASWGGGGKGI RKVHNDDEVR ALFKQVQGEV
 351  PGSPIFVMRL ASQSRHLEVQ LLCDQYGNVA ALHSRDCSVQ RRHQKIIEEG
 401  PVTVAPRETV KALEQAARRL AKAVGYVGAA TVEYLYSMET GDYYFLELNP
 451  RLQVEHPVTE WIAEVNLPAA QVAVGMGIPL WQIPEIRRFY GMDYGGGYDI
 501  WRKTAALATP FNFDEVDSQW PKGHCVAVRI TSEDPDDGFK PTGGKVKEIS
 551  FKSKPNVWAY FSVKSGGGIH EFADSQFGHA FAYGLSRPAA ITNMSLALKE
 601  IQIRGEIHSN VDYTVDLLNA SDFRENKIHT GWLDTRIAMR VQAERPPWYI
 651  SVVGGALYKT VTTNAATVSE YVSYLTKGHI PPKHISLVNS TVNLNIEGSK
 701  YTIETVRTGH GSYRLRMNDS TVEANVQSLC DGGLLMQLDG NSHVIYAEEE
 751  AGGTRLQIDG KTCLLQNDHD PSKLLAETPC KLLRFLVADG AHVDADVPYA
 801  EVEVMKMCMP LLSPASGVIH CMMSEGQALQ AGDLIARLDL DDPSAVKRAE
 851  PFDGIFPQME LPVAVSSQVH KRYAASLNAA RMVLAGYEHN INEVVQDLVC
 901  CLDNPELPFL QWDELMSVLA TRLPRNLKSE LEDKYKEYKL NFYHGKNEDF
 951  PSKLLRDIIE ENLSYGSEKE KATNERLVEP LMNLLKSYEG GRESHAHFVV
1001  KSLFEEYLTV EELFSDGIQS DVIETLRHQH SKDLQKVVDI VLSHQGVRNK
1051  AKLVTALMEK LVYPNPGGYR DLLVRFSSLN HKRYYKLALK ASELLEQTKL
1101  SELRASVARS LSDLGMHKGE MSIKDNMEDL VSAPLPVEDA LISLFDYSDR
1151  TVQQKVIETY ISRLYQPHLV KDSIQMKFKE SGAITFWEFY EGHVDTRNGH
1201  GAIIGGKRWG AMVVLKSLES ASTAIVAALK DSAQFNSSEG NMMHIALLSA
1251  ENESNISGIS SDDQAQHKME KLSKILKDTS VASDLQAAGL KVISCIVQRD
1301  EARMPMRHTF LWLDDKSCYE EEQILRHVEP PLSTLLELDK LKVKGYNEMK
1351  YTPSRDRQWH IYTLRNTENP KMLHRVFFRT IVRQPNAGNK FRSAQISDAE
1401  VGCPEESLSF TSNSILRSLM TAIEELELHA IRTGHSHMYL CILKEQKLLD
1451  LIPFSGSTIV DVGQDEATAC SLLKSMALKI HELVGARMHH LSVCQWEVKL
1501  KLDCDGPASG TWRVVTTNVT GHTCTIDIYR EVEEIESQKL VYHSASSSAG
1551  PLHGVALNNP YQPLSVIDLK RCSARNNRTT YCYDFPLAFE TALQKSWQSN
1601  GSTVSEGNEN SKSYVKATEL VFAEKHGSWG TPIIPMERPA GLNDIGMVAW
1651  IMEMSTPEFP NGRQIIVVAN DITFRAGSFG PREDAFFETV TNLACERKLP
1701  LIYLAANSGA RIGIADEVKS CFRVGWSDEG SPERGFQYIY LTEEDYARIS
1751  SSVIAHKLEL DSGEIRWIID SVVGKEDGLG VENIHGSAAI ASAYSRAYEE
```

FIG. 14

```
1801  TFTLTFVTGR TVGIGAYLAR LGIRCIQRLD QPIILTGFSA LNKLLGREVY
1851  SSHMQLGGPK IMATNGVVHL TVPDVLEGVS NILRWLSYVP ANIGGPLPIT
1901  KPLDPPDRPV AYIPENTCDP RAAICGVDDS QGKWLGGMFD KDSFVETFEG
1951  WAKTVVTGRA KLGGIPVGVI AVETQTMMQI IPADPGQLDS HERSVPRAGQ
2001  VWFPDSATKT AQALLDFNRE GLPLFILANW RGFSGGQRDL FEGILQAGST
2051  IVENLRTYNQ PAFVYIPMAG ELRGGAWVVV DSKINPDRIE CYAERTAKGN
2101  VLEPQGLIEI KFRSEELQDC MGRLDPELIN LKAKLQDVNH GNGSLPDIEG
2151  IRKSIEARTK QLLPLYTQIA IRFAELHDTS LRMAAKGVIK KVVDWEESRS
2201  FFYKRLRRRI AEDVLAKEIR QIVGDKFTHQ LAMELIKEWY LASQATTGST
2251  GWDDDDAFVA WKDSPENYKG HIQKLRAQKV SHSLSDLADS SSDLQAFSQG
2301  LSTLLDKMDP SQRAKFVQEV KKVLD
```

FIG. 14 (continued)

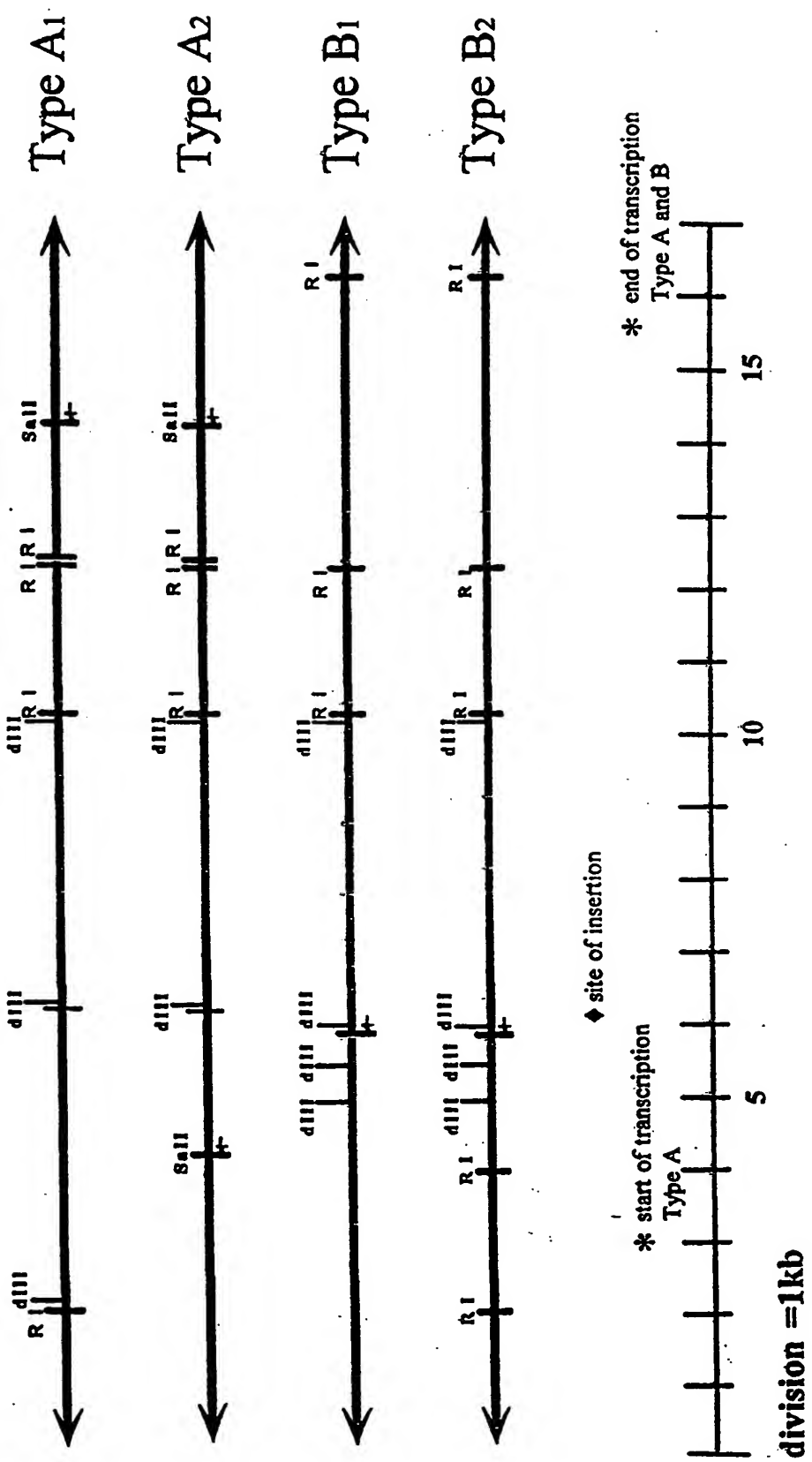
Type A1
Type A2
Type B1
Type B2
R
dIII
R I
SalI
site of insertion
* start of transcription
Type A
* end of transcription
Type A and B
division =1kb
5
10
15

FIG. 15

```
   1  AAGCTTGGTA TGGATTCgTC AGCGCCAAgC CGGGGTTTTG CATGCGCCCg
  51  ACTGGaArCs GAATTCCgTg AgCCCtGTaC rrCaATGGCA ACCCCAsGGT
 101  TACTggGGTG GCTGAATGGT CTCsGCTTAC GCAATTGTTT GTGGCAgCwG
 151  CGTGGGCTAA ATGTArGTTG TCTCTTGTTG CACTGCArGA TGGATGGGTA
 201  gCCTCTGGGC CGCCTCTGCT ArTGTCTArC GtTTGCTGAC TGTGGTTTAt
 251  TCAgGGATGC CCATGCCCAT GCTAgATTGA tAGGTGCCAT TCTAATGGTA
 301  GGTGGCGGTA AGGTTTATTA AGCTGyAGyA TCAGTAGGTA ACCTCATGAA
 351  TCAGGGTTTA aGCACACCTT TTCCTTTGTG TGGGTGCATA AGGAATGCAC
 401  TTGGCTTCGT TCCCTGATAG TCTTTGsTCA TGTGTCATTC TACCAAGTGG
 451  GTTACTGTAA CATTGCACTC TATGATGGTT GGTGGTtGTG CATCTTTyTG
 501  CTyCCCCTGG yTGTCTAATA CCTGCATGTa ACTGATGACC yyCyTTTATG
 551  TATCATATAG ATTACATCCT TTTGTTGTAC ATCTCAATTC TGAAAAAACA
 601  ATGTTTTGCA TTCTTAGCGc TCTGTGCaCA AgGaAAaGGa gGTTTTACCT
 651  gCAAcTtTTT TTTTCGAGAA AAAACAAACC TTTCTGAAaG gCAGTGATCA
 701  TTTAGtATAA AGAAAATTTG ATTTACTTTC TTCAGAGAGA AtATkCCAAr
 751  CAAACAATTT TCTTACTGTC TGAGCCACGA AATTTGATCT TGATCtTACT
 801  TTCACAAGCC ACATGAAGCC tTATCATCGC TcTGATAAAA AArCCAAaTA
 851  GGTGATTCAT AGAATGAGar AAAGAACCTG TTGCCATTTG GGGaCCTTGT
 901  TGTGTACTCA TTATCCCCCC TGCTCAGGTT GAGGTTTCCT TGCCACTGCC
 951  ACCCCTTGGC CCCTTCTTAT ACAACCATCT CCATTGAAAa AGATTTTGCA
1001  CtACATTTGG GCTTcGTATG aCAAAAaAGG aAAATaAAaC TaAaCAGCAG
1051  AAACATAGTA TAaTTATAGg TAAAAGGTTc TGGCAAGTTT GAGTGGaAGA
1101  GACCTTTGTA TATTTGGACA TATTTCACTA GTAAATAGTT TTCTAAAATc
1151  TTCATGAATG GTGGCCAATA AACTTGATAA GATCTCAACA TGGCAGGTTC
1201  CTTCmAAATG AGAGGAAAAC TGGAAACATC ACAAATATTT TTTAGCGAGT
1251  GGCCTATAAA TTATAATGTT GCTTTCATTT CTTTGATATT CaAAACTTCC
1301  TAAGAGTATT CTGCTAGAGC TCTGATGGTG TCTTTTGCCT CTGTCAGATT
1351  TTCCAGGaGT TTTCTTCCCT TTTtATGGCA CTGTGCGTTT GAGAAGGTCT
1401  TCAATTGTGC TGTCTGGGCC ACGGAACGAC AATGTCACAG CTTGGATTAG
1451  CCGCAGCTGC CTCAAAGGCC TTGCCACTAC TCCCTAATCG GCAGAGAAGT
1501  TCAGCTGGGA CTACATTCTC ATCATCTTCA TTATCGAGGC CCTTAAACAG
1551  AAGGAAAAGC CATACTCGtT CACTCCGTGA TGGCGGAGAT GGgGTATCAG
1601  ATGCCAAAAA GCACAGCCAG TCTGTTCGTC AAGGTACTGt GAATATCTTT
1651  tGaTACAAgc tAAAATTTtG cTACAGAATA TaTAtTtaAA GAGTtCTtTC
1701  TTGGcTGGtG ttGtTTATTT GttT.aACAt sCGAAAgGGC CtctAgttGg
1751  attGgttaGG tggsCTGAAT ACCACTCCTT AAGGTCTTGA GTTTGCTTTT
```

FIG. 16

```
1801  CCCCnCGGAG CGAATTTTAG GCTAGGGTTA CCCCCCCACC CCCACCCGAA
1851  TCTGCACAGy CCGGyCGyGG yCGyCCTCAT ATAGGCTACG ATGTCATTGT
1901  GTATCGGCGG GCCAGGGGTT TAAGAGTTTT CTTGACCTTT GTTAGAAGAT
1951  CTTAATAATA CAATGTCCAA GGGCTGTCTT ACCCTGTAGG TCGAGTTTTT
2001  AGTTGTTTTA ACATGGTAAT GTTTGAAGCC TCATTCTAGG TrCCAATATA
2051  GATATGCTCA CTGCTCAGTT TCAAATGTTT GTCTGCATGT AGGTCTTGCT
2101  GGCATTATCG ACCTCCCAAG TGAGGCACCT TCCGAAGTGG ATATTTCACA
2151  GTAAGGACTA CAATATTTTG CGTACGTTTG TTTTGGAAAA AGAAAATATT
2201  CTCAGCTTAT TTATACTAGC TTCGCTAATA CTGAAATGCT GTCTTAATGT
2251  CCTGGTGCTG TATGCTCAAT CTTTCATAGT AAATGCTGCA AAATATGTGA
2301  TGTAACTGTT GCAACACAGC CAGGGACCTG TTATTTAGAG CATGGTGAAT
2351  GCTCTGGTTC AGTTATATGA TGTAGTTATA GCTCATGTTG AAGAATTAGT
2401  TGCAGTGTTT GCTGGACAAT GGTCACTTAT TATAAATCAT ATCTGCATAC
2451  ACATTTGTGA CTTCTGTTGC TGTAAATGCC CGCATTTTTT GAGAAAAATT
2501  TAAATGCTTG GCCTAAATTG GACATATATG ATAGACAAAG CTGATTTGAA
2551  CTTTGTTTAT TTTTGACATC CATGCATATT GTCAGTGTTG TGAAAACAAT
2601  ACTAATCCTT TTTTTTTGTC TTTTTCCAGT GGATCTGAGG ATCCTAGGGG
2651  GCCAACAGAT TCTTATCAAA TGAATGGGAT TATCAATGAA ACACATAATG
2701  GAAGACATGC CTCAGTGTCC AAGGTTGTTG AATTTTGTGC GGCACTAGGT
2751  GGCAAAACAC CAATTCACAG TATATTAGTG GCCAACAATG GAATGGCAGC
2801  ACCCAAATTT ATGAGGAGTG TCCGGACATG GGCTAATGAT ACTTTTGGAT
2851  CTGAGAAGGC AATTCAACTC ATAGCTATGG CAACTCCGGA AGACATGAGG
2901  ATAAATGCAG AACACATTAG AATTGCTGAC CAATTACGTA GAGGTGCCTG
2951  GTGGAACAAA CAATAATAAC TACGCCAATG TTCAACTCAT AGTGGAGGTT
3001  AGCCTTGCTA ATCTGTTAGT TTACTACTGG TCTGCTGTTT CCTTTATTTG
3051  TTGTATAATG ATTGACATAT TTAAGTAGAG AAATTTATAT TTCTCCTCTG
3101  CTGTTGTGGA AGTCCAATTG TCATCATTAA CTGTGAAATA TTGCAGATGG
3151  CACAAAAACT AGGTGTTTCT GCTGTTTGGC CTGGTTGGGG TCATGCTTCT
3201  GAGAATCCTG AACTGCCAGA TGCATTGACC GCAAAAGGGA TCGTTTTTCT
3251  TGGCCCACCT GsATCATCAA TGAATGCTTT GGGAGATAAG GTCGGCTCAG
3301  CTCTCATTGC TCAAGCAGCC GGGGnCCCAA CTCTTGCTTG GAGTGGATCA
3351  CATGTGAGTC TCACTCTTTG ATTACTATCC GCCTGTCTCA TTGCTCTCTC
3401  TTTCATATTC TAATGACACT AAATTTAGGT TGAAGTTCCA TTAGAGTGCT
3451  GCTTAGACGC GATACCTGAG GAGATGTATA GAAAAGCTT
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FIG. 16 (continued)

```
   1 GAATTCCGTG AGCCCTGTAC GGCAATGGCA ACCCCAGGGT TACTGGGGTG
  51 GCTGAATGGT CTCGGCTTAC GCAATTGTTT GTGGCAGCTG CGTGGGCTAA
 101 ATGTAGGTTG TCTCTTGTTG CACTGCAGGA TGGATGGGTA GCCTCTGGGC
 151 CGCCTCTGCT AGTGTCTAGC GTTGCTGACT GTGGTTTATT CAGGGATGCC
 201 CATGCCCATG CTAGATTGAT AGGTCATAGG TGCCATTCTA ATGGTAGGTG
 251 GCGGTAAGGT TTATTAAGCT GTCGTATCAG TAGGTAACCT CATGAATCAG
 301 GGTTTAAGCC CACCTTCTCC TTTGTGTGGG TGCATAAGGA ATGCACTTGG
 351 CTTCGTTCCC TGCTAGTCTT TGCTCATGTG TCATTCTACC AAGTGGGTTA
 401 CTGTAACATT GCACTCTATG ATGGTTGGTG GTTGTGCATC TTTTTGCTTC
 451 CCCTGGTTGT CTAATACCTG CATGTAACTG ATGACCTTCT TTTATGTATC
 501 ATATAGATTA CATCTTTTGT TGTACATCTC AATTCTGAAA AACAATGTTT
 551 TGCATTCTTA GCGCTCTGTG CACAAGGAAA AGGAGGTTTT ACCTGCAACT
 601 TTTTTTTTCG AGAAAAAACA AACCTTTCTG AAAGGCAGTG ATCATTTAGT
 651 ATAAAGAAAA TTTGATTTAC TTTCTTCAGA GAGAATATTC CAAACAAACA
 701 ATTTTCTTAC AGTCTGAGCC ACGAAATTTG ATCTTGATCT TACTTTCACA
 751 AGCCACATGA AGCCTTATCA TCGCTCTGAT AAAAAAACCA AATAGGTGAT
 801 TCATAGAATG AGAAAAAGAA CCTGTTGCCA TTTGGGGACC TTGTTGTGTA
 851 CTCATTATCC CCCCTGCTCA GGTTGAGGTT TCCTTGCCAC TGCCACCCCT
 901 TGGCCCCTTC TTATACAACC ATCTCCATTG AAAAAGATTT TGCACTACAT
 951 TTGGGCTTCG TATAACAAAA AAGGAAAATA AAACTAAACA GCAGAAACAT
1001 AGTATAATTA TAGGTAAAAG GTTNTGGCAA GTTTGAGTGG TAGAGACCTT
1051 TGTATATTTG GACATATTTC ACTAGTAAAT AGTTTTCTAA AATGTTCATG
1101 AATGGTGGCC AATAAACTTG ATAAGATCTC AACATGGCAG GTTCCTTCAA
1151 AATGAGAGGA AAACTGGAAA CATCACAAAT ATTTTTTAGC GAGTGGCCTA
1201 TAAATTATAA TGTTGCTTTC ATTTCTTTGA TATTCAAAAC TTCCTAAGAG
1251 TATTCTGCTA GAGCTCTGAT GGTGTCTTTT GCCTCTGTCA GATTTTCCAG
1301 GAGTTTTCTT CCCTTTTTAT GGCACTGT
```

FIG. 17

```
   1  AGCATCCCTT GGGATTGTGA TnACTCACAT AAATTCTTGC GAAnTGTTGA
  51  CATTCTAGTG ATTTGAGTTC CGTTCTAGTG TGCTAGTCAn TTGAGCTCAA
 101  GTCTTGGTTT TATGTGTGCG TATTCACTGT GATCTTTGTG TCGTGTGTGA
 151  GTTGTTGATC CTTCCCTTGC TCCGTGATTC TTTGTGAAAT CTTTTGAAAG
 201  GGCGAGAGGC TCCAAGCTGT GGAGATTCCT CGCAAGTGGG ATTAAGAAAA
 251  GCAAAGCAAC ACCGTGGTAT TCAAGTTGGT CTTTGGACGG CTTGAGAGGG
 301  GTTGATTGCA ACCCTCGTCC GTTGGGACGC CACAACGTGG AGTAGGCAAG
 351  CGTTGGTCTT GGCCGAACCA GGGGATAACC ACCGTGCCAT CTCTGTGATT
 401  GATATCTCTT GGTTATTGTG TTGTGTTGAG ATCCTTCTCT AGCCACTTGG
 451  CAAATTACTG TGCTAACAAT TAATCAAGTT TTGTGGCTTA AGATTTTGAA
 501  GTATTACAGG ATCTGCATCA TGGTCTGTGT CTCCACAGCT ATGACACCCA
 551  CAGGAATTCA TGTGTTCCTT GGAGCCACTC TTGGATGACC TAAAGGAATT
 601  ATTTCTAACC GGCTTGTACA CATATGATGC ATCAAGAGAT GAGTGTTTTA
 651  CTATGCGAGG GGCCATGCTT ATGACCATAA GTAATCTTCC TGGTTTAGAA
 701  ATGCTTGCTT CTCATATGGT TCATGGGAAA TTCGCATGCC TCCTTGTGGT
 751  GAAAATGTCT GGACAAAACA GCTGAAGAAT GGTCGTAAAT CTTGTTTTAT
 801  GGGAAATCGC CAATATATTG ATCTTGATCA TTCTTATTGC TTGGATGCAG
 851  ACTCCGTTTG ATGGAACGAT AGACTTCGAA CAAAACCTAA AACCTATTAT
 901  GATCGTCCAA TTTTGGATGA AATCATCACA CTTGGTGATT TCAAGAACTC
 951  AAAAAyTTAC AGTTAATTGG ATATAGGAGG GnGCAAAAAC ACAGTAAGTT
1001  GGACATTCCA TAAGGGGATT TATTTTAGTT GACAATAAAG TAGATGGGCA
1051  TCATCCTGAG TTTnGTTTGG CATCGTGTCG TAGATTGAAA CTGTAAGGAT
1101  GGACATGGTA GnTAACAGGT TGAGATGAAT GATTCAACAG TTGAAGCGAA
1151  TGTACAATCT TTATGTGATG GTGGCTnTTA ATGCAGGTAA CTAGTTTTTT
1201  TTTATGCTTT ATTATTAATT AGTTGGATAA ATGGTTTnGA TTTnTGATTG
1251  TTAAAnTGCA ATGGCTCCAG TTGGATGGnA ACAGCCAnGT AATTTATGCA
1301  GAAGnAGnAG nTGGTGGTAC ACGGnTTCAG ATTGATGGAA AGAnATGTTT
1351  ATTGCAGGTA AATAnTCCCT TnTTCCTTTA TATTTTTGTT GTnTGATTGT
1401  ATAAnTTTGn TAGATTATTT GTATAATTTA TTATTGCATT TCACCCCACT
1451  AAnTTATTTT TAAAAGATGG GTTTTGTTGT TTGnTTCAGC nGGCGACATC
1501  ACATAAGnAA ATTGTGATTA ATTTTTGTTT TTTTGCAGnA TGACCATGAT
1551  CCATCAAAGT TATTA
```

FIG. 18A

```
   1  ACATAAGCTG GGTTAGTAGT GGTGAATTAG TGGATTATTG ATCTGTTGTG
  51  GGCAAGGAGn TGGCTTGGTG TCGAGAAAAA ACATGGAaGT GTGTATTGCC
 101  AGTGcTTaTT yTrGGGATAT GAGGGAATTw AmATTAcATT TGTGAcTGGG
 151  CGGACTGTAG GATAGGAGTT ATCTTGyTcG ATTGGTATAC GGTGCATACA
 201  GskyTTGACC AGCTATTATT TTAACAGGgT TTTCTGCCCT GAACAAGTCC
 251  TTGGGCGGGA AGTGTACAGc TCCCACATGC AGCTTGGTGG TCCTAAGATC
 301  ATGGCGACCA ATGGTGTTGT CCACCTCACT GTTCCAGATG ACCTTGAAGG
 351  TGTTTCCAAT ATATTGAGGT GGCTCAGCTA TGTTCCTGCA AACATTGGTG
 401  GACCTcTTCC TATTACCAAA CCTCTGGACC CTCCAGACAG ACCTGTTGCT
 451  TACATCCCTG AGAACACATG CGATGCACGT GCAGCTATCT GTGGTGTAGA
 501  TGACAGCCAA GGGAAATGGT TGGGTGGTAT GTTTGACAAA GACAGCTTTG
 551  TGGAGACATT TGAAGGATGG GCAAAAACAG TGGTTACTGG CAGAGCAAAG
 601  CTTGGAGGAA TTCCATGCAT CTTAATAAAC ACAGTTGGCC CTTAAAGCAA
 651  GTGAACTTCT TGAACAAACC AAACTAAGTG AACTCCGTGC AAGCGTTGCA
 701  AGAAGCCTTT CGGATCTGGG GATGCATAAG GGAGAAATGA GTATTAAGGA
 751  TAACATGGAA GATTTAGTCT CTGCCCCATT ACCTGTTGAA GATGCTCTGA
 801  TTTCTTTGTT TGATTACAGT GATCGAACTG TTCAGCAGAA AGTGATTGAG
 851  ACATACATAT CACGATTGTA CCAGGTATTA TATCAACTAA CTTAATGTCT
 901  TCCATAGTCT CACTAAGCAT ATCTGATATG tTTAGATACC CTACATGGAA
 951  TGCTCATCTT TTCATTTGAC ACAAAGAAAC ATTGAGAAAT GAGATGCTGA
1001  CGATTGGCTG AAATTAACTG GGTnTGAGAA ATTGTGATCT CCCAACTTGT
1051  TAATGCACAA TGTTCTGGCT AACTTGCCAA TATTTTTTCA GCCTCATCTT
1101  GTTnAGGATA GCAnCCAAAT GAAATCCAAG GATCTGGTGC TATTACTTTT
1151  TGGGAATTTA TGAAGGGC
```

FIG. 18B

```
  1  CTCCCAATAT TGTCATGAGG CTTGCATCCC AGGTTAGTTT TTTTTCCTTT
 51  CTGAAATTTA TATTCCATAC CTTTTCAGCT TTAGTTATCC TTGTATTTTC
101  TGGAAGCTTC ATCTGATGCA TTATTGACAA ATGCACTAAT GGTCATCATA
151  TTTGGAGATT AACATATTTA TCTTAATTGA TGGGAACTCT TGAAAATGAC
201  AATGGTTGAG CAGATAATTA ACAGTTTTTT AATAAAAAAA CATGCATTTC
251  TAGGAGTTGG ACTAAGCTTT TCTTAGTATG AAGTGCCATG TTTTACATGG
301  TCCATTTGTG TCAATTTACA GTCGGTATCA TGGAAAGGTT GTCATAATGG
351  CTGGAGANAA ACAACACATC TTGTTTCTCA ACACTTGTGG GAGAAGANGT
401  TTTACCTTTT TTCCTAAAAT TACTTTTTGT ACTAAATTGT ATAATTTTTC
451  CAATATTCTC CATGATTATT GAACTCTGCT GTGTTCAAAC AGCCAAAACA
501  TGTTTCCATA CTTTACACCT TTATTTTTTA GATGGAACCT GGAATTGTGC
551  TCTGTTATCT GTATCATGCA TATATTGATC TTAAACCTAT CTCTATTGTA
601  GAATCCGCAC TTGAATTCAG TTGCTTGTGA TCAATATG
```

FIG. 18C

```
  1  GGTAACCACC ACACCCGCGG CGCTTAATGG CCGTACAGGG NGGTCCCATT
 51  CGCCATTCAG GTGCGCAACT GTTGGGAAGG GCGATCGGTG CGGGCTTCTT
101  CGNTATTACG CCAGCTGGCG AAAGGGGGAT GTGCTGCAAG GCGATTAAGT
151  TGGGTAACGC CAGGGTTTTC CCAGTCACGA CGTTGTAAAA CGACGGCCAG
201  TGAGCGCGCG TAATACGACT CACTATAGGG CGAATTGGGT ACCGGGCCCC
251  CCCTCGAGGT CGACCTGCAG GTCAACGGAT CCTAGGGGGC CAACAGATTC
301  TTATCAAATG AATGGGATTA TCAATGAAAC ACATAATGGA AGACATGCCT
351  CAGTGTCCAA GGTTGTTGAA TTTTGTGCGG CACTAGGTGG CAAAACACCA
401  ATTCACAGTA TATTAGTGGC CAACAATGGA ATGGCAGCAG CAAAATTTAT
451  GAGGAGTGTC CGGACATGGG CTAATGATAC TTTTGGATCT GAGAAGGCAA
501  TTCAACTCAT AGCTATGGCA ACTCCGGAAG ACATGAGGTA AATGCAGAAC
551  ACATTAGA
```

FIG. 18D

```
   1  GAATAATCTG CCTGCAGCTC AAGTTGCTGT TGGAATGGGC ATACCTCTTT
  51  GGCAGATTCC AGGTAATTAC CAATTTACCA ACTTATTTAG TTCCTTATTG
 101  TTTTATTCTC TAATTTTCTA CTTATGTAgA AATCAGACGT TTCTATGGAA
 151  TGGACTATGG AGGAGGGTAT GACATTTGGA GGAAAACAGC AGCTCTTGCT
 201  ACACCATTTA ATTTTGATGA AGTAgATTcT CAATGGCCAA AGGGCCATTG
 251  TGTAgCAGTT AGAATTACTA GTGAGGACCC AGATGATGGT TTCAAACcTA
 301  CTGGTGGGAA AGTGAAGGTA AGTTTTCTAG ATGACATGTA TTATATATCG
 351  TTCAAAgAgA TTAAGTTTGG TTAAATGAcT AGGTCTTGAT TTTTTATCTT
 401  TCAGGAGATA AGTTTTAAAA GCAAGCCTAA TGTTTGGGCC TaCTTCTCAG
 451  TAAAGGTaAC TTGTTAACTT TAGTACGCTG TCACATTATt ctTCsTTGTG
 501  .AAAATAAtT TGAACGGTtC TCTTTGTATT TTaACCAtCC AtCgTCTCAT
 551  TTAsCAgAgC ACACAAATAT TtGCACTGAC GCCCcTcCCC tTATCtGCtT
 601  TCAgTCTGGT GGAgGCATtC AtGAATTtGC TGATTCTCAG TTCGGTATGT
 651  GTAAACCAAG AGTATTCTTT GTAATTTATA TTGGTCCTCA ATTTTGAAAT
 701  ATTGcTCTTT CCGTTACAGG ACAwGTTTTT GCATATGGGC TCTCTAGATC
 751  AGCAGCAATA ACAAACATGA CTCTTGCATT AAAAnAGATT CAAATTCGTG
 801  GAGAAATTCA TTCAAATGTT TGATTACACA GTTGATCTCT TAAATGTTAA
 851  GAAATATTAA CCACCTTTTA AATCACATTT TCCATTATGT TTGATTGCAT
 901  ATCATTAATT TTGATTTTCT ATTATGGCTA AACCTGTGGT GCTATTTTCC
 951  TATTATCCCA GGCTTCCGAC TTTAGA
```

FIG. 18E

```
                                                    GGATCC TAgGGGGCCA
1401  ACAGATTCTT ATCAAATGrA aTGGGATTAT CAATGAAACA CATAATGGAA
1451  GACATGCCTC AGTGTCCAAG GTTGTTGAAT TTTGTGCGGC ACTAGGTGGC
1501  AAAACACCAA TTCACAGTAT ATTAGTGGCC AACAATGGAA TGGCAGCAgC
1551  aAAATTTATG AggAGTGTCC GGACATGGGC TAATGATACT TTTGGATCTG
1601  AGAAgGCAAT TCAACTCATA GCTATgGCAA CTCCGGAAGA CATGAGGATA
1651  AATGCAGAAC ACATTAGAAT TGCTGACCAA TTaCGTAgAr gTGcctgGTG
1701  gaacaaACAA tA.ATAActA cgCCaATGTT CAAcTcATAg TGGAaGTTAG
1751  CCTTGcTAAT CTGTTAgTTT ACTACTGgTC tGCtGTTtCC TTtATTtGtt
1801  GTaTAATGAT tGACaTATTt AagTAgAgAA atTTATAtTT CtCctCtgCt
1851  GTTGTGgAag TCCAatTGTC acCATtAACt GTgAAaTATt gCAgATgGca
1901  CaAAAACtAG gTgtTTCTgC TGTTTGGCCT gGTTGGGGTC ATGCTTCTGA
1951  GAATCcTGAA CTGCCAGATG CATTGACCGC AAAAGGGATC GTTTTTCTTG
2001  GCCCACCTGc ATCATCAATG AATGCTTTGG GAGATAAGGT CGGCTCAGCT
2051  CTCATTGCTC AAGCAGGCGG GGtCCCAACT CTTGCTTGGA GTGGATCACA
2101  TGTGAGTCTC ACTCTTTGAT TACTATCCGC CTGTCTCATT GCTCTCTCTT
2151  TCATATTCTA ATGACACTAA ATTTAGGTTG AAGTTCCATT AGAGTGCTGC
2201  TTAGACGCGA TACCTGAGGA GATGTATAGA AAAGCTT
```

FIG. 18F

```
  1  AATTCCTGTG GGTGTTATAG CTGTGGAGAC ACAGACCATG ATGCAGCTCA
 51  TCCCTGCTGA TCCAGGTCAA CTTGATTCCC ATGAGCGATG TGTTCCTCGG
101  GCTGGACAAG TGTGGTTCCC AGATNCTGCA ACCAAGACAG CTCAGGCATT
151  ATTAGACTTC AACCGTGAAG GATTGCCTCT GTTCATCCTG GCTAACTGGA
201  GAGGCTtCTC TGGGGgACAG AGAGATCTCT T
```

FIG. 19A

```
  1  AATTCATGCA TCTTAATAAA CACAGTTGGC CCTTAAAGCA AGTGAACTTC
 51  TTGAACAAAC CAAACTAAGT GAACTCTGTT CCAGCATTGC AAGAAGCCTT
101  TCAGATCTGG GGATGCATAA GGGAGAAATG ACTATTAAGG ATAGCATGGA
151  AGATTTAGTC TCTGNCCCAT TGCCTGTTGA AGATGCTCTT ATTTCTTTGT
201  TTGATTA
```

FIG. 19B

```
  1  ATAGACCTGT CGCATACATC CCTGAGAACA CATGCGATCC GCGTGCAGCC
 51  ATCCGTGGnG TAGATGACAG CCAAGGGAAA TGGTTGGGTG GTATGTTTGA
101  CAAAGACAGC TTTGTGGAGA CATTTGAAGG ATGGGCAAAA ACAGTGGTTA
151  CTGGTAGAGC AAAGCTTGGA GGAAGGAATT
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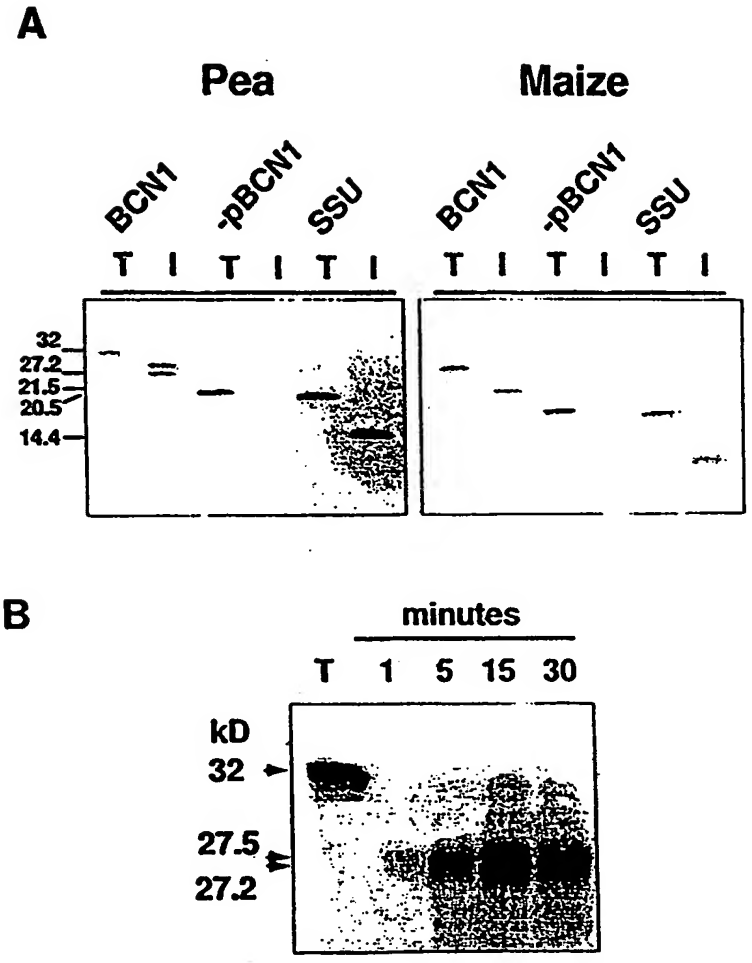
FIG. 19C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BCN1
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SSU
T I T I T I T I T I T I
32
27.2
21.5
20.5
14.4
B
minutes
T 1 5 15 30
kD
32
27.5
27.2

FIG.20

1 ACAAAATAGT AAAGACCTCC AGAAGGTTGT AGACATTGTT TTGTCTCACC

51 AGGGTGTGAG AAGCAAAACC AAGCTgAtAC TCaCGCtCAT gGAgAAACTG

101 GTCtATCCaA ACCCTgcTGC CtATAGGGaC CAGcTgATTC GCtTTTcTTC

151 CCTCAACCAT aAAAGaTaTT aCAAGcTGGC CCTTaAAgcT AgTgAACTTc

201 TcGaACaAAC CaAGcTCAgT gaACtCCGca CAAGCATTGT AaGGAaCcTT

251 tcAGCgCTGg AgATGTTTAC TGAAGAAAGG GCAGGTTTCT CGTTGCAAGA

301 GAAGAAATTG GCCATTAATG AGAGCATGGG AGATTTAGTC AGTGCCCCAC

351 TGCCAGTTGA AGATGCACTT ATATCTTTGT TTGATTGTAT TGATcAAACT

401 CTTCAGCAGA GAGTGATCGA GACGTACATA TCTCGATTAT ACCAGCCTcA

451 ACTTGTGAAG GATAGTATCC AGCTGAAATA TCAGGATTCT GCTGtTATCG

501 CTTTATGGGA ATTCAACGAA GGGCATCCTG ATAATAGATT GGGTGCTATG

551 GTTATCCTGA AGTCTCTAGA ATCTGTGTCA ACAGCAATTG GAGCTGCTCT

601 AAAGGATAGA TCACATTATG CAAGCTCTGC GGGTAACACA GTGCATATTG

651 TTTTGTTGGA TGCTGATACT CAGACGAATA CAACTGAAGA TAGTGGTGAT

701 AACGACCAAG CTCAAGACAG GATGGACAAA CTCTCTGTAA TACTGAAACA

751 AGATATTGTC ACGGCTGATC TATGTGCTGC TGGTGTCAAG GTTATTAGTT

801 GCATTGtTCA AAGAGATGGA GCACTCATGC CTATgCGCCG TACCTTCCTC

851 TTGTCTGAAG AAAAGCTTTG TTATGAGGAA GAGCCGATTC TTCGGCATGT

901 GGAGCCTCCA CTTTCTGCAC TTCTTGAGTT GGATAAATTG AAAGTGAAAG

951 GATaCAATGA GATGAAGTAT ACACCGTCAC GTGATCGTCA GTGGCATATA

1001 TACACACTTA GAAATACTGA AAACCCCAAA ATGTTGCACA GGGTATTTTT

1051 CCGAACTCTT GTCAGACAGC CGAGTGCTGG CAACAGGTTT ATGTCAGGCC

1101 ATATCAATGA TGTTGAAGTA GGACATGCTG AGGAATCTCT TTCATTTACA

1151 TCAAGCAGCA TATTAAGATC TTTGATGACT GCTATAGAAG AATTGGAGCT

1201 TCACGCGATT AGGACTGGTC ATTCTCATAT GTACTTGTGC ATATTGAAAG

1251 AGCAAAAGCT TCTTGACCTT GtTCCTGTTT CAgGGAACAC AGTTGTGGAT

1301 GTTGGCCAAG ATGAAGCTAC TGCGTGCTCT CTTATGAAAG AAATGGCTCT

1351 AAAGATACAT GAACTTGTTG GTGCAAGAAT GCATCATCTT TCTGtATGCC

1401 AGTGGGAAGT gAAACTTAAG TTGGACAGTG ATGGACCTGC CAGTGGTAGC

1451 TGGAGAGTTG TAACAACCAA TGTCACTAGT CACACCTGCA CTGTGGATAT

1501 CTACCGGGAG GTCGAAGATa CAGATTCACA GAAGCTAGTA TACCACTATG

Figure 21

1551 CTGCATCGTC ACCTGGTCCT TTGCATGGTG TCACATTGAG TAATTCATAC

1601 CAGCCTTTGA GCGTTATTGA TCTAAAACGA TGCTCTGCCA GGAACAACAG

1651 AACTACATAC TGCTACGATT TTCCATTGGC ATTTGAAACT GCAGTGCGGA

1701 AGTCATGGGC TAACGTTTCt AGTgAAAaCA aCCAATGTtA TGTTAAagCA

1751 aCAgAGcTGG TGTTtGCTgA CAAGAATGGG TCGTGGGGCA CTCCTATAGT

1801 TCCTATGCAG CGTGCTGCTG GGCTCAATGA CATCGGTATG GtTGCgTGGA

1851 TCtTGGaCAT GTCCaCTCCT GAATTTCCCA GTGGCAGACA GATTATTGTT

1901 ATCGCAAATG ATATTACTTT CAGAGCTGGA TCATTTGGCC CAAGGGAAGA

1951 TGCATTTTTT GAAGCTGTTA CCAACCTGGC TTGTGAGAGA AAGCTTCCTC

2001 TTATCTACAT GGCAGCAAAC TCTGGTGCTC GGATTGGCAT TGCTGATGAA

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2401 CTtAAcTgGG TTTTCTGCCc TGaACAAGCT TCTCGGGCGG gAAGTGTACA

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2501 GTCCATCTGA CTGTTCCAGA TGACCTTgAA GGTGtTtCtA ATATAtTGAG

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2701 GTTGGGTGGT ATGTTTGACA AAGACAGTTT TGTGGAGACA TTTGAAGGAT

2751 GGGCAAAGAC AGTAGTTACT GGCAGAGCAA AACTTGGAGG GATTCCTGTG

2801 GGTGTTATAG CTGTGGAGAC ACAGACCATG ATGCAGCTCG TCCCTGCAGA

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2901 TCTGGTTTCC AGATTCAGCT ACCAAGACAG CGCACGCAAT GTTGGACTTC

2951 AACCGTgAAG GATTACCTCT GTTCATCCTt GCTAAcTGGA GAGGCTTCTC

3001 TGGTGGGCAA AGAGACCTTT TTGAAGGAAT T

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51 LNHKRYYKLA LKASELLEQT KLSELRTSIV RNLSALEMFT EERAGFSLQE

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201 KDRSHYASSA GNTVHIVLLD ADTQTNTTED SGDNDQAQDR MDKLSVILKQ

251 DIVTADLCAA GVKVISCIVQ RDGALMPMRR TFLLSEEKLC YEEEPILRHV

301 EPPLSALLEL DKLKVKGYNE MKYTPSRDRQ WHIYTLRNTE NPKMLHRVFF

351 RTLVRQPSAG NRFMSGHIND VEVGHAEESL SFTSSSILRS LMTAIEELEL

401 HAIRTGHSHM YLCILKEQKL LDLVPVSGNT VVDVGQDEAT ACSLMKEMAL

451 KIHELVGARM HHLSVCQWEV KLKLDSDGPA SGSWRVVTTN VTSHTCTVDI

501 YREVEDTDSQ KLVYHYAASS PGPLHGVTLS NSYQPLSVID LKRCSARNNR

551 TTYCYDFPLA FETAVRKSWA NVSSENNQCY VKATELVFAD KNGSWGTPIV

601 PMQRAAGLND IGMVAWILDM STPEFPSGRQ IIVIANDITF RAGSFGPRED

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901 LGGMFDKDSF VETFEGWAKT VVTGRAKLGG IPVGVIAVET QTMMQLVPAD

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Figure 2a

Figure 3B

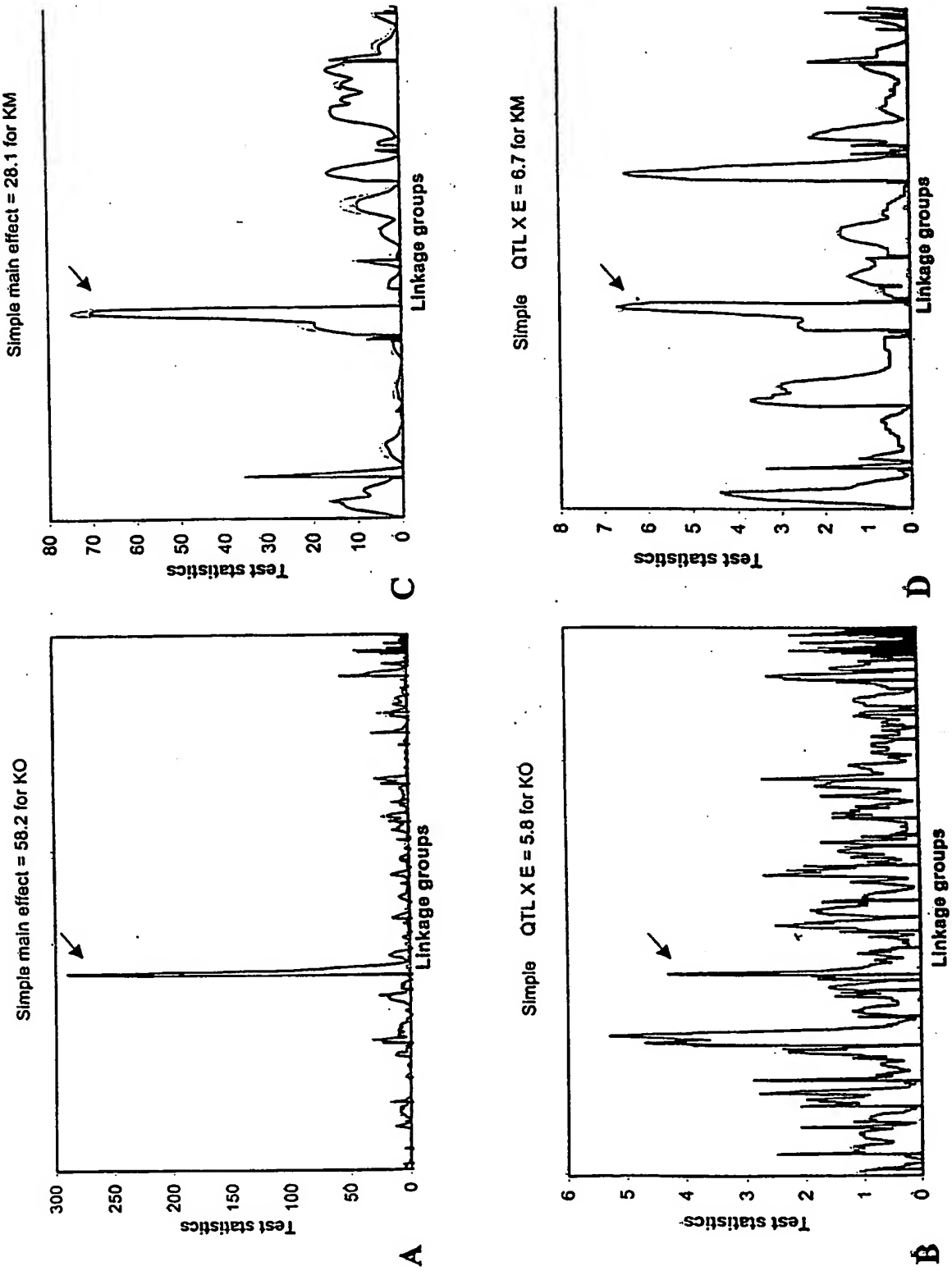
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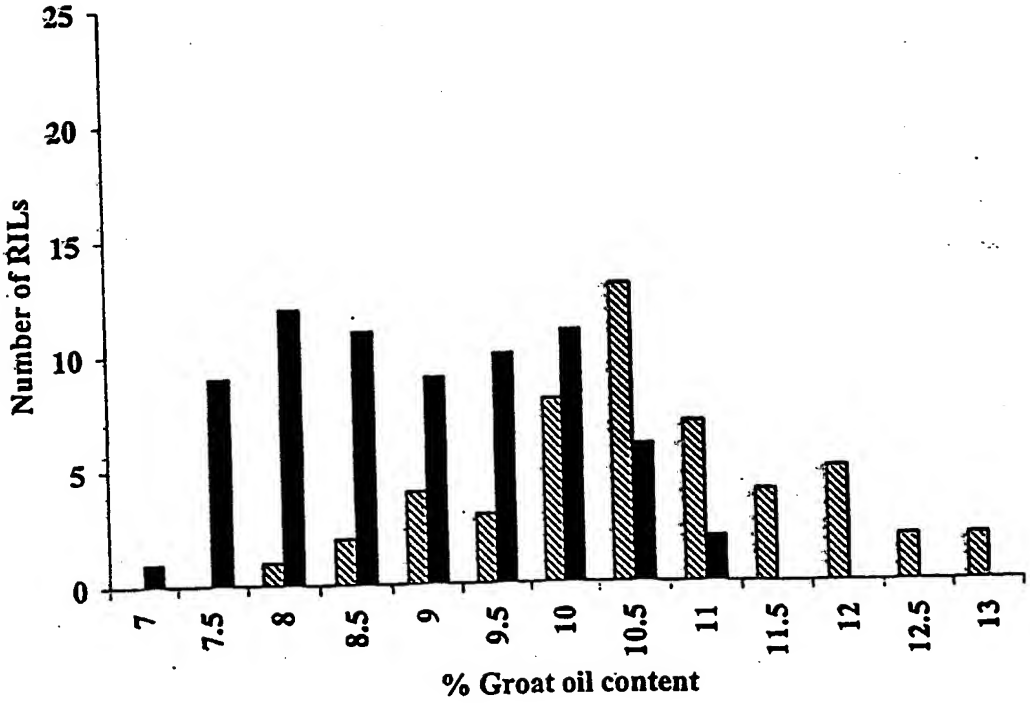
Figure 2a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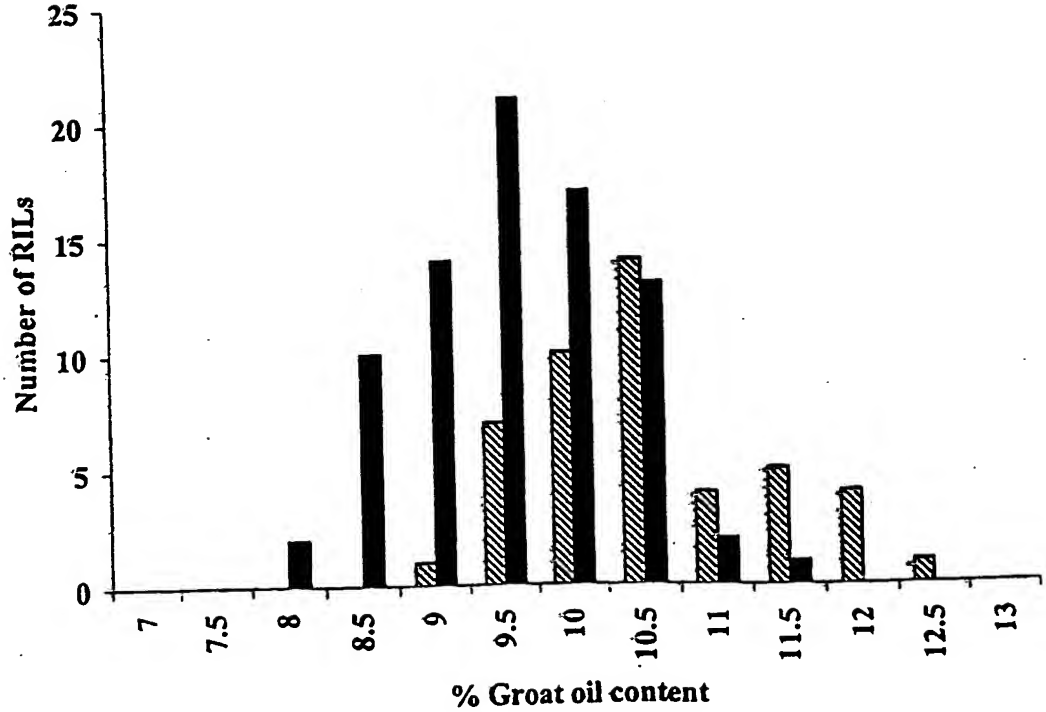
11.5
12
12.5
13
% Groat oil content

A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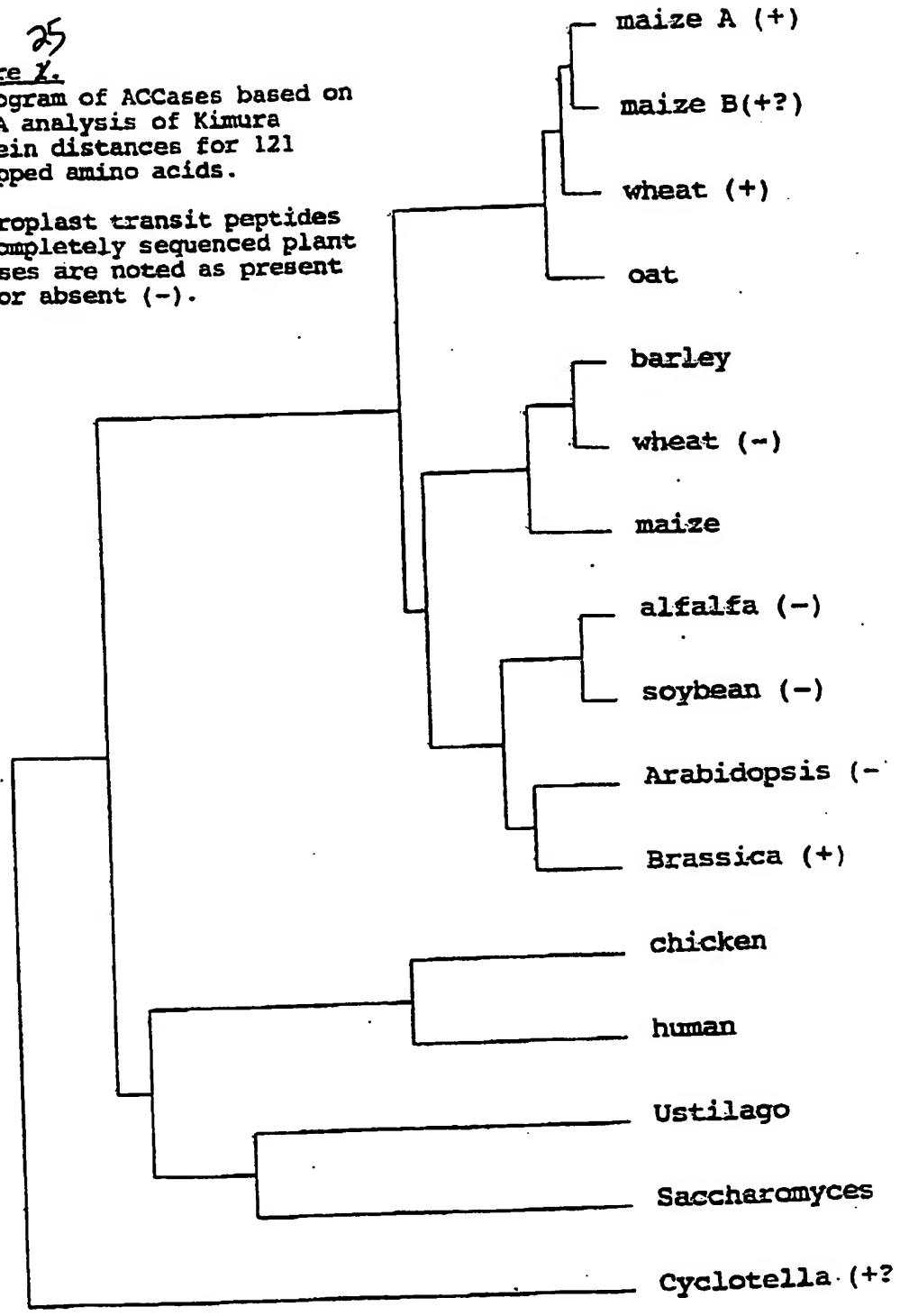
7
7.5
8
8.5
9
9.5
10
10.5
11
11.5
12
12.5
13
% Groat oil content

B

25

Figure X.
Phylogram of ACCases based on UPGMA analysis of Kimura protein distances for 121 ungapped amino acids.

-Chloroplast transit peptides on completely sequenced plant ACCases are noted as present (+) or absent (-).



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Lys Val Gly Ser Ala Leu Ile Ala Gln Ala Ala Gly Val Pro Thr Leu
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Gln Leu Asp Gly Asn Ser His Val Ile Tyr Ala Glu Glu Glu Ala Gly
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Phe Leu Val Ala Asp Gly Ala His Val Asp Ala Asp Val Pro Tyr Ala
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1730 1735 1740
Asp Tyr Ala Arg Ile Ser Ser Ser Val Ile Ala His Lys Leu Glu Leu
1745 1750 1755 1760
Asp Ser Gly Glu Ile Arg Trp Ile Ile Asp Ser Val Val Gly Lys Glu
1765 1770 1775
Asp Gly Leu Gly Val Glu Asn Ile His Gly Ser Ala Ala Ile Ala Ser
1780 1785 1790
Ala Tyr Ser Arg Ala Tyr Glu Glu Thr Phe Thr Leu Thr Phe Val Thr
1795 1800 1805
Gly Arg Thr Val Gly Ile Gly Ala Tyr Leu Ala Arg Leu Gly Ile Arg
1810 1815 1820
Cys Ile Gln Arg Leu Asp Gln Pro Ile Ile Leu Thr Gly Phe Ser Ala
1825 1830 1835 1840
Leu Asn Lys Leu Leu Gly Arg Glu Val Tyr Ser Ser His Met Gln Leu
1845 1850 1855
Gly Gly Pro Lys Ile Met Ala Thr Asn Gly Val Val His Leu Thr Val
1860 1865 1870
Pro Asp Val Leu Glu Gly Val Ser Asn Ile Leu Arg Trp Leu Ser Tyr
1875 1880 1885
Val Pro Ala Asn Ile Gly Gly Pro Leu Pro Ile Thr Lys Pro Leu Asp
1890 1895 1900
Pro Pro Asp Arg Pro Val Ala Tyr Ile Pro Glu Asn Thr Cys Asp Pro
1905 1910 1915 1920

Arg Ala Ala Ile Cys Gly Val Asp Asp Ser Gln Gly Lys Trp Leu Gly
1925 1930 1935
Gly Met Phe Asp Lys Asp Ser Phe Val Glu Thr Phe Glu Gly Trp Ala
1940 1945 1950
Lys Thr Val Val Thr Gly Arg Ala Lys Leu Gly Gly Ile Pro Val Gly
1955 1960 1965
Val Ile Ala Val Glu Thr Gln Thr Met Met Gln Ile Ile Pro Ala Asp
1970 1975 1980
Pro Gly Gln Leu Asp Ser His Glu Arg Ser Val Pro Arg Ala Gly Gln
1985 1990 1995 2000
Val Trp Phe Pro Asp Ser Ala Thr Lys Thr Ala Gln Ala Leu Leu Asp
2005 2010 2015
Phe Asn Arg Glu Gly Leu Pro Leu Phe Ile Leu Ala Asn Trp Arg Gly
2020 2025 2030
Phe Ser Gly Gly Gln Arg Asp Leu Phe Glu Gly Ile Leu Gln Ala Gly
2035 2040 2045
Ser Thr Ile Val Glu Asn Leu Arg Thr Tyr Asn Gln Pro Ala Phe Val
2050 2055 2060
Tyr Ile Pro Met Ala Gly Glu Leu Arg Gly Gly Ala Trp Val Val Val
2065 2070 2075 2080
Asp Ser Lys Ile Asn Pro Asp Arg Ile Glu Cys Tyr Ala Glu Arg Thr
2085 2090 2095
Ala Lys Gly Asn Val Leu Glu Pro Gln Gly Leu Ile Glu Ile Lys Phe
2100 2105 2110
Arg Ser Glu Glu Leu Gln Asp Cys Met Gly Arg Leu Asp Pro Glu Leu
2115 2120 2125
Ile Asn Leu Lys Ala Lys Leu Gln Asp Val Asn His Gly Asn Gly Ser
2130 2135 2140
Leu Pro Asp Ile Glu Gly Ile Arg Lys Ser Ile Glu Ala Arg Thr Lys
2145 2150 2155 2160
Gln Leu Leu Pro Leu Tyr Thr Gln Ile Ala Ile Arg Phe Ala Glu Leu
2165 2170 2175
His Asp Thr Ser Leu Arg Met Ala Ala Lys Gly Val Ile Lys Lys Val
2180 2185 2190
Val Asp Trp Glu Glu Ser Arg Ser Phe Phe Tyr Lys Arg Leu Arg Arg
2195 2200 2205
Arg Ile Ala Glu Asp Val Leu Ala Lys Glu Ile Arg Gln Ile Val Gly
2210 2215 2220
Asp Lys Phe Thr His Gln Leu Ala Met Glu Leu Ile Lys Glu Trp Tyr
2225 2230 2235 2240

Leu Ala Ser Gln Ala Thr Thr Gly Ser Thr Gly Trp Asp Asp Asp Asp
2245 2250 2255
Ala Phe Val Ala Trp Lys Asp Ser Pro Glu Asn Tyr Lys Gly His Ile
2260 2265 2270
Gln Lys Leu Arg Ala Gln Lys Val Ser His Ser Leu Ser Asp Leu Ala
2275 2280 2285
Asp Ser Ser Ser Asp Leu Gln Ala Phe Ser Gln Gly Leu Ser Thr Leu
2290 2295 2300
Leu Asp Lys Met Asp Pro Ser Gln Arg Ala Lys Phe Val Gln Glu Val
2305 2310 2315 2320
Lys Lys Val Leu Asp
2325

<210> 7
<211> 22
<212> DNA
<213> Zea mays

<400> 7
ggtcttcaat tgtgctgtct gg 22

<210> 8
<211> 24
<212> DNA
<213> Zea mays

<400> 8
ccttgacgaa cagactggct gtgc 24

<210> 9
<211> 23
<212> DNA
<213> Zea mays

<400> 9
cacagccagt ctgttcgtca agg 23

<210> 10
<211> 20
<212> DNA

<213> Zea mays

<400> 10
cctctacgta attggtcagc 20

<210> 11
<211> 20
<212> DNA
<213> Zea mays

<400> 11
catagctatg gcaactccgg 20

<210> 12
<211> 3488
<212> DNA
<213> Zea mays

<220>
<221> unsure
<222> (1803)...(1803)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (3324)...(3324)
<223> "n" represents "a" or "g" or "c" or "t"

<400> 12
aagcttggta tggattcgtc agcgccaagc cggggttttg catgcgcccg actggaarcs 60
gaattccgtg agccctgtac rrcaatggca accccasggt tactggggtg gctgaatggt 120
ctcsgcttac gcaattgttt gtggcagcwg cgtgggctaa atgtargttg tctcttgttg 180
cactgcarga tggatgggta gcctctgggc cgcctctgct artgtctarc gtttgctgac 240
tgtggtttat tcagggatgc ccatgcccat gctagattga taggtgccat tctaatggta 300
ggtggcggta aggtttatta agctgyagya tcagtaggta acctcatgaa tcagggttta 360
agcacacctt ttcctttgtg tgggtgcata aggaatgcac ttggcttcgt tccctgatag 420
tctttgstca tgtgtcattc taccaagtgg gttactgtaa cattgcactc tatgatggtt 480
ggtggttgtg catctttytg ctyccccctgg ytgtctaata cctgcatgta actgatgacc 540
yycytttatg tatcatatag attacatcct tttgttgtac atctcaattc tgaaaaaaca 600
atgttttgca ttcttagcgc tctgtgcaca aggaaaagga ggttttacct gcaacttttt 660
ttttcgagaa aaaacaaacc tttctgaaag gcagtgatca tttagtataa agaaaatttg 720

atttactttc ttcagagaga atatkccaar caaacaattt tcttactgtc tgagccacga 780
aatttgatct tgatcttact ttcacaagcc acatgaagcc ttatcatcgc tctgataaaa 840
aarccaaata ggtgattcat agaatgagar aaagaacctg ttgccatttg gggaccttgt 900
tgtgtactca ttatcccccc tgctcaggtt gaggtttcct tgccactgcc acccccttggc 960
ccctttcttat acaaccatct ccattgaaaa agattttgca ctacatttgg gcttcgtatg 1020
acaaaaaagg aaaataaaac taaacagcag aaacatagta taattatagg taaaaggttc 1080
tggcaagttt gagtggaaga gacctttgta tatttggaca tatttcacta gtaaatagtt 1140
ttctaaaatc ttcatgaatg gtggccaata aacttgataa gatctcaaca tggcaggttc 1200
cttcmaaatg agaggaaaac tggaaacatc acaaatattt tttagcgagt ggcctataaa 1260
ttataatgtt gctttcattt ctttgatatt caaaacttcc taagagtatt ctgctagagc 1320
tctgatggtg tcttttgcct ctgtcagatt ttccaggagt tttcttccct ttttatggca 1380
ctgtgcgttt gagaaggtct tcaattgtgc tgtctgggcc acggaacgac aatgtcacag 1440
cttggattag ccgcagctgc ctcaaaggcc ttgccactac tccctaatcg ccagagaagt 1500
tcagctggga ctacattctc atcatcttca ttatcgaggc ccttaaacag aaggaaaagc 1560
catactcgtt cactccgtga tggcggagat ggggtatcag atgccaaaaa gcacagccag 1620
tctgttcgtc aaggtactgt gaatatcttt tgatacaagc taaaattttg ctacagaata 1680
tatatttaaa gagttctttc ttggctggtg ttgtttattt gtttaacats cgaaagggcc 1740
tctagttgga ttggttaggt ggsctgaata ccactcctta aggtcttgag tttgcttttc 1800
cccncggagc gaattttagg ctagggttac ccccccaccc ccacccgaat ctgcacagyc 1860
cggycgyggy cgycctcata taggctacga tgtcattgtg tatcggcggg ccaggggttt 1920
aagagttttc ttgacctttg ttagaagatc ttaataatac aatgtccaag ggctgtctta 1980
ccctgtaggt cgagttttta gttgttttaa catggtaatg tttgaagcct cattctaggt 2040
rccaatatag atatgctcac tgctcagttt caaatgtttg tctgcatgta ggtcttgctg 2100
gcattatcga cctcccaagt gaggcacctt ccgaagtgga tatttcacag taaggactac 2160
aatattttgc gtacgtttgt tttggaaaaa gaaaatattc tcagcttatt tatactagct 2220
tcgctaatac tgaaatgctg tcttaatgtc ctggtgctgt atgctcaatc tttcatagta 2280
aatgctgcaa aatatgtgat gtaactgttg caacacagcc agggacctgt tatttagagc 2340
atggtgaatg ctctggttca gttatatgat gtagttatag ctcatgttga agaattagtt 2400
gcagtgtttg ctggacaatg gtcacttatt ataaatcata tctgcataca catttgtgac 2460
ttctgttgct gtaaatgccc gcatttttg agaaaaattt aaatgcttgg cctaaattgg 2520
acatatatga tagacaaagc tgatttgaac tttgtttatt tttgacatcc atgcatattg 2580
tcagtgttgt gaaaacaata ctaatccttt ttttttgtct ttttccagtg gatctgagga 2640
tcctaggggg ccaacagatt cttatcaaat gaatgggatt atcaatgaaa cacataatgg 2700
aagacatgcc tcagtgtcca aggttgttga attttgtgcg gcactaggtg gcaaaacacc 2760
aattcacagt atattagtgg ccaacaatgg aatggcagca cccaaattta tgaggagtgt 2820
ccggacatgg gctaatgata cttttggatc tgagaaggca attcaactca tagctatggc 2880
aactccggaa gacatgagga taaatgcaga acacattaga attgctgacc aattacgtag 2940
aggtgcctgg tggaacaaac aataataact acgccaatgt tcaactcata gtggaggtta 3000
gccttgctaa tctgttagtt tactactggt ctgctgtttc ctttatttgt tgtataatga 3060
ttgacatatt taagtagaga aatttatatt tctcctctgc tgttgtggaa gtccaattgt 3120

catcattaac tgtgaaatat tgcagatggc acaaaaacta ggtgtttctg ctgtttggcc 3180
tggttggggt catgcttctg agaatcctga actgccagat gcattgaccg caaaagggat 3240
cgtttttctt ggcccacctg satcatcaat gaatgctttg ggagataagg tcggctcagc 3300
tctcattgct caagcagccg gggncccaac tcttgcttgg agtggatcac atgtgagtct 3360
cactctttga ttactatccg cctgtctcat tgctctctct ttcatattct aatgacacta 3420
aatttaggtt gaagttccat tagagtgctg cttagacgcg atacctgagg agatgtatag 3480
aaaagctt 3488

<210> 13
<211> 1328
<212> DNA
<213> Zea mays

<220>
<221> unsure
<222> (1024)...(1024)
<223> "n" represents "a" or "g" or "c" or "t"

<400> 13
gaattccgtg agccctgtac ggcaatggca accccagggt tactggggtg gctgaatggt 60
ctcggcttac gcaattgttt gtggcagctg cgtgggctaa atgtaggttg tctcttgttg 120
cactgcagga tggatgggta gcctctgggc cgcctctgct agtgtctagc gttgctgact 180
gtggtttatt cagggatgcc catgcccatg ctagattgat aggtcatagg tgccattcta 240
atggtaggtg gcggtaaggt ttattaagct gtcgtatcag taggtaacct catgaatcag 300
ggtttaagcc caccttctcc tttgtgtggg tgcataagga atgcacttgg cttcgttccc 360
tgctagtctt tgctcatgtg tcattctacc aagtgggtta ctgtaacatt gcactctatg 420
atggttggtg gttgtgcatc tttttgcttc ccctggttgt ctaatacctg catgtaactg 480
atgaccttct tttatgtatc atatagatta catcttttgt tgtacatctc aattctgaaa 540
aacaatgttt tgcattctta gcgctctgtg cacaaggaaa aggaggtttt acctgcaact 600
tttttttcg agaaaaaaca aacctttctg aaaggcagtg atcatttagt ataaagaaaa 660
tttgatttac tttcttcaga gagaatattc caaacaaaca attttcttac agtctgagcc 720
acgaaatttg atcttgatct tactttcaca agccacatga agccttatca tcgctctgat 780
aaaaaaacca aataggtgat tcatagaatg agaaaaagaa cctgttgcca tttggggacc 840
ttgttgtgta ctcattatcc ccctgctca ggttgaggtt tccttgccac tgccacccct 900
tggccccttc ttatacaacc atctccattg aaaaagattt tgcactacat ttgggcttcg 960
tataacaaaa aaggaaaata aaactaaaca gcagaaacat agtataatta taggtaaaag 1020
gttntggcaa gtttgagtgg tagagacctt tgtatatttg gacatatttc actagtaaat 1080
agttttctaa aatgttcatg aatggtggcc aataaacttg ataagatctc aacatggcag 1140
gttccttcaa aatgagagga aaactggaaa catcacaaat atttttagc gagtggccta 1200
taaattataa tgttgctttc atttctttga tattcaaaac ttcctaagag tattctgcta 1260

gagctctgat ggtgtctttt gcctctgtca gattttccag gagttttctt ccctttttat 1320
ggcactgt 1328

<210> 14
<211> 1565
<212> DNA
<213> Zea mays

<220>
<221> unsure
<222> (22)...(22)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (44)...(44)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (90)...(90)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (982)...(982)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1064)...(1064)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1112)...(1112)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1177)...(1177)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1238)...(1238)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1244)...(1244)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1256)...(1256)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1279)...(1279)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1288)...(1288)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1305)...(1305)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1308)...(1308)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1311)...(1311)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1325)...(1325)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1344)...(1344)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1365)...(1365)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1372)...(1372)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1393)...(1393)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1405)...(1405)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1410)...(1410)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1453)...(1453)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1484)...(1484)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1491)...(1491)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1508)...(1508)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1539)...(1539)
<223> "n" represents "a" or "g" or "c" or "t"

<400> 14
agcatccctt gggattgtga tnactcacat aaattcttgc gaantgttga cattctagtg 60
atttgagttc cgttctagtg tgctagtcan ttgagctcaa gtcttggttt tatgtgtgcg 120
tattcactgt gatctttgtg tcgtgtgtga gttgttgatc cttcccttgc tccgtgattc 180

tttgtgaaat cttttgaaag ggcgagaggc tccaagctgt ggagattcct cgcaagtggg 240
attaagaaaa gcaaagcaac accgtggtat tcaagttggt ctttggaccg cttgagaggg 300
gttgattgca accctcgtcc gttgggacgc cacaacgtgg agtaggcaag cgttggtctt 360
ggccgaacca cgggataacc accgtgccat ctctgtgatt gatatctctt ggttattgtg 420
ttgtgttgag atccttctct agccacttgg caaattactg tgctaacaat taatcaagtt 480
ttgtggctta agattttgaa gtattacagg atctgcatca tggtctgtgt ctccacagct 540
atgacaccca caggaattca tgtgttcctt ggagccactc ttggatgacc taaaggaatt 600
atttctaacc ggcttgtaca catatgatgc atcaagagat gagtgtttta ctatgcgagg 660
ggccatgctt atgaccataa gtaatcttcc tggtttagaa atgcttgctt ctcatatggt 720
tcatgggaaa ttcgcatgcc tccttgtggt gaaaatgtct ggacaaaaca gctgaagaat 780
ggtcgtaaat cttgttttat gggaaatcgc caatatattg atcttgatca ttcttattgc 840
ttggatgcag actccgtttg atggaacgat agacttcgaa caaaacctaa aacctattat 900
gatcgtccaa ttttggatga aatcatcaca cttggtgatt tcaagaactc aaaaayttac 960
agttaattgg atataggagg gngcaaaaac acagtaagtt ggacattcca taaggggatt 1020
tattttagtt gacaataaag tagatgggca tcatcctgag tttngtttgg catcgtgtcg 1080
tagattgaaa ctgtaaggat ggacatggta gntaacaggt tgagatgaat gattcaacag 1140
ttgaagcgaa tgtacaatct ttatgtgatg gtggctntta atgcaggtaa ctagtttttt 1200
tttatgcttt attattaatt agttggataa atggtttnga tttntgattg ttaaantgca 1260
atggctccag ttggatggna acagccangt aatttatgca gaagnagnag ntggtggtac 1320
acggnttcag attgatggaa aganatgttt attgcaggta aatantccct tnttccttta 1380
tatttttgtt gtntgattgt ataantttgn tagattattt gtataattta ttattgcatt 1440
tcaccccact aanttatttt taaaagatgg gttttgttgt ttgnttcagc nggcgacatc 1500
acataagnaa attgtgatta atttttgttt ttttgcagna tgaccatgat ccatcaaagt 1560
tatta 1565

<210> 15
<211> 1168
<212> DNA
<213> Zea mays

<220>
<221> unsure
<222> (60)...(60)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1024)...(1024)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure

<222> (1104)...(1104)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (1114)...(1114)
<223> "n" represents "a" or "g" or "c" or "t"

<400> 15

acataagctg ggttagtagt ggtgaattag tggattattg atctgttgtg ggcaaggagn 60
tggcttggtg tcgagaaaaa acatggaagt gtgtattgcc agtgcttatt ytrgggatat 120
gagggaattw amattacatt tgtgactggg cggactgtag gataggagtt atcttgytcg 180
attggtatac ggtgcataca gskyttgacc agctattatt ttaacagggt tttctgccct 240
gaacaagtcc ttgggcggga agtgtacagc tcccacatgc agcttggtgg tcctaagatc 300
atggcgacca atggtgttgt ccacctcact gttccagatg accttgaagg tgtttccaat 360
atattgaggt ggctcagcta tgttcctgca aacattggtg gacctcttcc tattaccaaa 420
cctctggacc ctccagacag acctgttgct tacatccctg agaacacatg cgatccacgt 480
gcagctatct gtggtgtaga tgacagccaa gggaaatggt tgggtggtat gtttgacaaa 540
gacagctttg tggagacatt tgaaggatgg gcaaaaacag tggttactgg cagagcaaag 600
cttggaggaa ttccatgcat cttaataaac acagttggcc cttaaagcaa gtgaacttct 660
tgaacaaacc aaactaagtg aactccgtgc aagcgttgca agaagccttt cggatctggg 720
gatgcataag ggagaaatga gtattaagga taacatggaa gatttagtct ctgccccatt 780
acctgttgaa gatgctctga tttctttgtt tgattacagt gatcgaactg ttcagcagaa 840
agtgattgag acatacatat cacgattgta ccaggtatta tatcaactaa cttaatgtct 900
tccatagtct cactaagcat atctgatatg tttagatacc ctacatggaa tgctcatctt 960
ttcatttgac acaaagaaac attgagaaat gagatgctga cgattggctg aaattaactg 1020
ggtntgagaa attgtgatct cccaacttgt taatgcacaa tgttctggct aacttgccaa 1080
tatttttca gcctcatctt gttnaggata gcanccaaat gaaatccaag gatctggtgc 1140
tattactttt tgggaattta tgaagggc 1168

<210> 16
<211> 638
<212> DNA
<213> Zea mays

<220>
<221> unsure
<222> (358)...(358)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure

<222> (398)...(398)
<223> "n" represents "a" or "g" or "c" or "t"

<400> 16
ctcccaatat tgtcatgagg cttgcatccc aggttagttt tttttccttt ctgaaattta 60
tattccatac cttttcacct ttagttatcc ttgtattttc tggaagcttc atctgatgca 120
ttattgacaa atgcactaat ggtcatcata tttggagatt aacatattta tcttaattga 180
tgggaactct tgaaaatgac aatggttgag cagataatta acagtttttt aataaaaaaa 240
catgcatttc taggagttgg actaagcttt tcttagtatg aagtgccatg ttttacatgg 300
tccatttgtg tcaatttaca gtcggtatca tggaaaggtt gtcataatgg ctggaganaa 360
acaacacatc ttgtttctca acacttgtgg gagaagangt tttacctttt ttcctaaaat 420
tacttttgt actaaattgt ataatttttc caatattctc catgattatt gaactctgct 480
gtgttcaaac agccaaaaca tgtttccata ctttacacct ttatttttta gatggaacct 540
ggaattgtgc tctgttatct gtatcatgca tatattgatc ttaaacctat ctctattgta 600
gaatccgcac ttgaattcag ttgcttgtga tcaatatg 638

<210> 17
<211> 558
<212> DNA
<213> Zea mays

<220>
<221> unsure
<222> (41)...(41)
<223> "n" represents "a" or "g" or "c" or "t"

<221> unsure
<222> (103)...(103)
<223> "n" represents "a" or "g" or "c" or "t"

<400> 17
ggtaaccacc acacccgcgg cgcttaatgg ccgtacaggg nggtcccatt cgccattcag 60
gtgcgcaact gttgggaagg gcgatcggtg cgggcttctt cgntattacg ccagctggcg 120
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50 55 60

Glu Leu Leu Glu Gln Thr Lys Leu Ser Glu Leu Arg Thr Ser Ile Val
65 70 75 80

Arg Asn Leu Ser Ala Leu Glu Met Phe Thr Glu Glu Arg Ala Gly Phe
85 90 95

Ser Leu Gln Glu Lys Lys Leu Ala Ile Asn Glu Ser Met Gly Asp Leu
100 105 110

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Ser Thr Ala Ile Gly Ala Ala Leu Lys Asp Arg Ser His Tyr Ala Ser
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Ser Ala Gly Asn Thr Val His Ile Val Leu Leu Asp Ala Asp Thr Gln
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INTERNATIONAL SEARCH REPORT

International Application No
PC /US 99/14022

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N9/00 C12N15/82 A01H5/00 A01H5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL EBI Accession No: 048959, 1 June 1998 (1998-06-01) GORNICKI P. ET AL.: "ACETYL-COENZYME A CARBOXYLASE; from Triticum Aestivum" XP002122229 see sequence abstract --- -/--	1-50

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 November 1999	26/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Chakravarty, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL EBI Accession No. Q08367, 1 November 1996 (1996-11-01) ASHTON A.R. ET AL.: "ACETYL-COA CARBOXYLASE (ACC); from Zea Mays" XP002122230 see sequence abstract	1-50
X	DATABASE EMBL EBI Accession no. AF029895, 3 February 1998 (1998-02-03) GORNICKI P. ET AL.: "Triticum aestivum acetyl-coenzyme A carboxylase (Acc-1) mRNA, nuclear gene encoding plastid protein, complete cds." XP002122231 see sequence abstract	1-50
X	DATABASE EMBL EBI Accession no. U58598, 31 May 1996 (1996-05-31) ASHTON A.R. ET AL.: "Zea mays acetyl CoA carboxylase mRNA, partial cds." XP002122232 see sequence abstract	1-50
X	WO 93 11343 A (GAHAN JOHN PETER) 10 June 1993 (1993-06-10) figure 3	1-50
X	WO 96 31609 A (UNIV MINNESOTA ;GENGENBACH BURLE G (US); SOMERS DAVID A (US); WYSE) 10 October 1996 (1996-10-10) the whole document	1-50

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
Pc./US 99/14022

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9311343 A	10-06-1993	AU 2937192 A	28-06-1993
WO 9631609 A	10-10-1996	BR 9608013 A	02-03-1999
		CA 2217367 A	10-10-1996
		EP 0820513 A	28-01-1998